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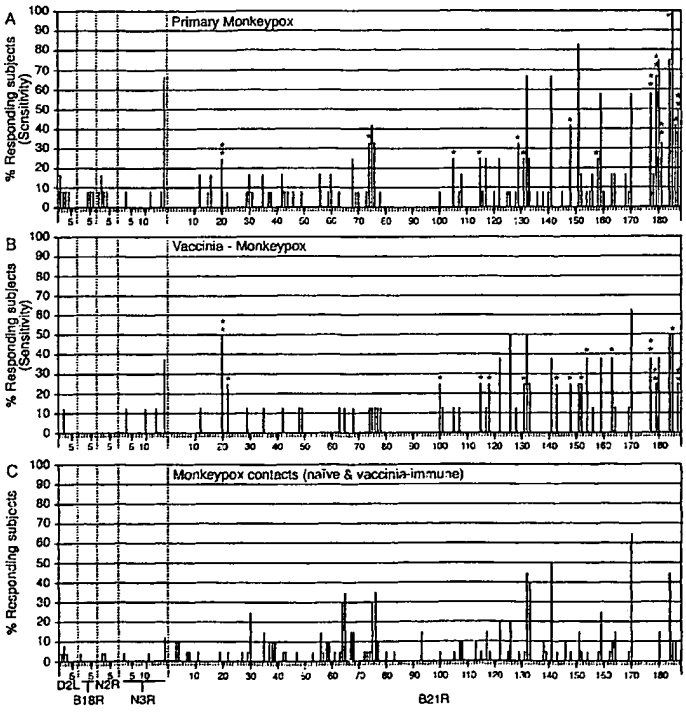
(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF ORTHOPOXVIRUSES



(57) Abstract: In particular aspects, the invention provides a novel approach for the systematic analysis and identification of biologically relevant epitopes (SABRE). SABRE-identified polypeptides have diagnostic (e.g., polypeptide arrays, etc.) and/or therapeutic (e.g., vaccines, etc.) utility, and utility for developing monoclonal antibodies having diagnostic and/or therapeutic utility (e.g. for detecting and/or preventing orthopoxvirus infection). Preferred aspects provide high-throughput assays for detecting specific orthopoxvirus infection, for detecting orthopoxvirus-specific immune response, or for dual (parallel) determination of both orthopoxvirus immune response and orthopoxvirus infection. Additional preferred and surprising aspects provide novel high-throughput methods for detecting 'protective immunity' against orthopoxviruses (e.g., for detecting protective immunity against smallpox virus and monkeypox virus), based on anti-vaccinia virus serum antibody levels. The inventive diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

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COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF ORTHOPOXVIRUSES

FIELD OF THE INVENTION

The invention generally relates generally to orthopoxviruses (*e.g.*, smallpox, vaccinia and monkeypox), and more particularly to diagnostic and therapeutic methods comprising use of orthopoxvirus proteins, polypeptides and anti-orthopoxvirus antibodies. Additionally, the invention relates to novel methods for systematic analysis of biologically relevant epitopes (SABRE™).

CROSS-REFERENCE TO RELATED APPLICATIONS

The application claims the benefit of priority to United States Provisional Patent Application Serial Number 60/579,048, filed 12 June 2004, which is incorporated by reference herein in its entirety.

BACKGROUND

Orthopoxviruses. Orthopox viruses, including smallpox, monkeypox and vaccinia viruses, cause a number of contagious infections, and can be fatal. Smallpox, for example, is a highly contagious, often fatal disease caused by variola virus. About 30% of those infected with the smallpox virus die. Smallpox outbreaks had occurred periodically for thousands of years. Fortunately, naturally occurring smallpox virus was eliminated worldwide in 1978 through the outstanding efforts of the WHO Global Eradication Program. Nonetheless, there is an ongoing concern that terrorists, or rogue nations or states might be able to obtain, or potentially create, a deposit of smallpox and develop a biological weapon of mass destruction. Such concerns are legitimate for several reasons.

Scientists have retained stocks of the variola virus for research purposes in two secure laboratories, one at the CDC in Atlanta, Georgia, and the other in Moscow, Russia. The CDC has classified smallpox as the highest priority (Category A) bioterrorism threat to the U.S. public health system and national security due to the fact that variola virus can be easily disseminated and transmitted from person to person, has the potential to cause widespread

illness and death, and requires special actions for public health preparedness. Additionally, there is currently no specific treatment for smallpox disease, and the only prevention is vaccination.

Moreover, and significantly, the last mass vaccination was in the mid 1970's, and through this highly successful vaccination program, >90% of Americans over the age of 35 (~140 million people; 2000 U.S. Census Bureau) have already been vaccinated against smallpox. Nonetheless, current views on smallpox immunity suggest that residual immunity against smallpox and vaccinia is questionable, being low or non-existent in today's population, because vaccination using vaccinia virus for immunization against smallpox occurred many years ago (roughly 25 to 75 years ago.

Prior art detection of orthopoxviruses. The ability to rapidly respond to a potential outbreak initially depends upon the availability of assays suitable for rapid and specific detection of the condition or agent before substantial communication thereof. Preferably, such assays should be virus specific, and should allow for detection of exposure to orthopoxvirus before the active stages of the disease; that is, prior to formation of skin lesions.

PCR-based assays. While very sensitive PCR-based detection methods for orthopoxviruses are available, these assays have significant disadvantages. One disadvantage is that PCR assays require specialized equipment and uncontaminated reagents, and, in the orthopoxvirus context, are typically performed in a limited number of specialized centers. Such PCR-based assays are thus not readily available as facile 'first response'-type 'field' assays systems. Furthermore, PCR techniques detect specific polynucleotides that are present during viral replication, and are thus only effective in active stages of the disease; that is, when skin lesions are showing. This is a relatively narrow time window, and thus false-negative results may be obtained. For example, during a recent monkeypox outbreak in Wisconsin, there was at least one case where a person, who owned a prairie dog that had died of a monkeypox virus infection, but who tested negative for the monkeypox virus by the PCR-based assay. This individual had all of the standard clinical symptoms of a monkeypox infection including pox lesions, but failed to go to the hospital during the early stages of the

disease. While an ELISA test showed that this person was infected by the monkeypox virus, the PCR-based assay failed to detect the virus.

Plaque-reduction assays. In practice, the vaccinia plaque-reduction test can be used to determine the serum dilution at which 50% of the infectious virus (*e.g.*, vaccinia) is neutralized (NT_{50}). The disadvantage of this assay, however, are that it is time consuming, cumbersome and cannot be used as a rapid, high-throughput platform. Historically, the vaccinia plaque-reduction test was employed for determining anti-smallpox immunity by indirectly measuring the levels of vaccinia-specific neutralizing antibodies in the serum.

ELISA. Currently, rapid and relatively facile ELISA-based assays are available, in some cases, to quantify virus-specific Ig levels. However, orthopoxvirus-specific ELISA platforms do not exist for all orthopoxviruses (*e.g.*, monkeypox). Additionally, as widely recognized in the art, ELISA assays of serum antibodies are uniformly regarded as not having utility for determination of *protective immunity*.

In summary, while very sensitive PCR-based assays exist, they are applicable over a relatively narrow window of infection, and are not suited to 'first response'-type 'field' conditions. Moreover, while plaque-reduction tests are available, they are cumbersome and not suited for rapid, high-throughput conditions. Furthermore, while ELISA-based assays are available, they are regarded as having no utility for determination of protective immunity, and are not specific, in some cases to a particular virus (*e.g.*, as in the case of monkeypox virus).

Therefore, there is a pronounced need in the art for reliable and efficient methods for the detection of viral infection, including detecting a viral infection during all stages, rather than detecting the virus only when it is in its replicative stage.

There is a pronounced need in the art for reliable and efficient methods for *dual* or parallel detection of monkeypox virus (MPV) infection, and MPV-specific immune response.

There is a pronounced need in the art for reliable and efficient detection of protective immunity against orthopoxviruses, including smallpox.

There is a pronounced need in the art for novel anti-MPV antibodies, and antibody compositions comprising anti-MPV antibodies, and methods of treatment and prevention using anti-MPV antibodies and/or compositions comprising anti-MPV antibodies.

SUMMARY OF THE INVENTION

In particular aspects, the invention provides a novel approach for systematic analysis of biologically relevant epitopes (SABRE) having substantial utility for rapidly and effectively mapping biologically relevant peptide epitopes suitable for novel diagnostic and/or therapeutic applications.

Particular embodiments provide for using the SABRE-identified polypeptides to develop monoclonal antibodies, and compositions comprising such antibodies, having substantial utility as novel diagnostic reagents for detecting the respective pathogen (*e.g.* for detecting orthopoxvirus infection). The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Additional embodiments provide for using SABRE-identified polypeptides to develop monoclonal antibodies, and compositions comprising such antibodies, for novel therapeutic use for treatment or prevention of orthopoxvirus (*e.g.*, smallpox, monkeypox and vaccinia) infections, comprising using the inventive antibodies and antibody compositions to treat an infection, to alleviate symptoms of the infection, and/or to help prevent pathogen infection.

Yet additional embodiments provide vaccines, based on the use of one or more SABRE-identified antigens in vaccine compositions.

Further embodiments provide for using the SABRE-identified polypeptides to develop novel high-throughput assays for the detection of orthopoxvirus-specific immune response, based on measurement of orthopoxvirus-specific serum antibody levels. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Yet additional embodiments provide for using the SABRE-identified polypeptides and respective antibodies in high-throughput methods for *dual* (parallel) determination of orthopoxvirus immune response and orthopoxvirus infection. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Further embodiments provide for using SABRE-identified polypeptides and respective antibodies in high-throughput methods for determination of orthopoxvirus-specific (*e.g.*, smallpox-specific, monkeypox-specific, smallpox/monkeypox-specific) immune response and orthopoxvirus infection.

Yet further embodiments provide an array of different orthopoxvirus (*e.g.*, monkeypox virus) peptide epitopes coupled to a solid phase.

In yet additional aspects, the present invention represents a surprising departure from the long-standing art-recognized dogma that particular immunological (*e.g.*, ELISA) assays have no utility for determination of *protective immunity* against orthopoxviruses, and particular embodiments provide rapid and reliable high-throughput methods for detecting protective immunity against orthopoxviruses (*e.g.*, for determination of protective immunity against smallpox virus, based on anti-vaccinia virus serum antibody levels. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the levels of Virus-specific $CD4^+$ T cell memory following smallpox vaccination.

Figures 2A and B show the levels of virus-specific $CD8^+$ T cell memory following smallpox vaccination.

Figure 3A, 3B and 3C show the relationship between vaccinia-specific $CD4^+$ and $CD8^+$ T cell memory over time. Comparisons were made between the number of antiviral $CD4^+$ and $CD8^+$ T cells from the same individual. Figure 3A, 3B, and 3C show 1 month to 7 years post-vaccination (p.v.), 14 to 40 years p.v., and 41 to 75 years p.v., respectively.

Figures 4A, 4B and 4C show long-lived antiviral antibody responses induced by smallpox vaccination. Figure 4A, 4B, and 4C show the quantitation of vaccinia-specific antibody responses by ELISA (4A), the levels of vaccinia-specific antibody titers (1 to 75 years post-vaccination) compared to the total number of vaccinations received (4B), and the correlation between virus-specific antibody titers and neutralizing antibodies (4C), respectively.

Figures 4D, 4E and 4F show the relationship between virus-specific $CD4^+$ (closed symbols) or $CD8^+$ (open symbols) T cells (per million $CD4^+$ or $CD8^+$ T cells, respectively) with virus-specific antibody titers as determined at 1 month to 7 years post-vaccination (p.v.) (4D), 14 years to 40 years p.v. (4E), and 41 years to 75 years p.v. (4F), respectively.

Figures 5A-5D show antiviral antibody responses following orthopoxvirus infection (see EXAMPLE V herein below).

Figure 6 shows diagnosis of recent monkeypox infection by quantitation of orthopoxvirus-specific T cells. The frequency of virus-specific T cells capable of producing both IFN□ and TNF□ after direct *ex vivo* stimulation with vaccinia virus was determined by intracellular cytokine staining (ICCS).

Figure 7 shows analysis of monkeypox-specific peptide ELISA assays for diagnosing monkeypox infection. Serum or plasma samples (1:50 dilution) obtained at 2 months to 1 year post-infection/exposure were incubated on ELISA plates coated with an individual peptide in each well. Samples were scored positive for a particular peptide if they scored ≥2-fold over background on at least 2 to 3 different ELISA plates.

Figure 8 shows the relationship between reported and unreported (i.e. asymptomatic) monkeypox infections. This figure was modified from a similar flow-chart diagram published by Reed *et al.* (11) and shows the relationship between different monkeypox survivors in the context of the WI monkeypox outbreak. Patients 4 and 5 are subjects who purchased 39 prairie dogs from an Illinois distributor and sold 2 prairie dogs to the family in the Northwestern WI household, the site of the first recorded case of human monkeypox in the United States.

FIGURE 9 shows a comparison of the number of monkeypox lesions reported by unvaccinated and vaccinated monkeypox patients. Subjects were asked to fill out a medical history questionnaire describing their history of monkeypox infection including the number of monkeypox lesions or "pocks" that developed during the course of this acute viral infection.

DETAILED DESCRIPTION OF THE INVENTION

In particular aspects, the invention provides a novel approach, herein referred to as SABRE, for systematic analysis of biologically relevant epitopes of pathogen proteins. SABRE provides for rapid and effective mapping and identification of biologically relevant peptide epitopes of pathogen proteins that are suitable for novel diagnostic and/or therapeutic applications. Preferred pathogen proteins are those of the orthopoxviruses (*e.g.*, smallpox, vaccinia and monkeypox). The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

SABRE-identified polypeptides have utility for developing respective antibodies (*e.g.*, monoclonal antibodies), and compositions comprising such antibodies, having utility as novel diagnostic reagents for detecting the respective pathogen (*e.g.* for detecting orthopoxvirus infection). The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

SABRE-identified polypeptides have utility for developing antibodies (*e.g.*, monoclonal antibodies), and compositions comprising such antibodies, having therapeutic utility for treatment or prevention of orthopoxvirus (*e.g.*, smallpox, monkeypox and vaccinia) infections. The inventive antibodies and antibody compositions have utility for treating an infection, for alleviating symptoms of an infection, and/or to prevent pathogen infection.

The SABRE-identified polypeptides provide vaccines, based on the use of one or more SABRE-identified antigens in vaccine compositions.

The SABRE-identified polypeptides were used herein to develop novel high-throughput assays for the detection of orthopoxvirus-specific *immune response*, based on measurement of orthopoxvirus-specific serum antibody levels. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Additionally, according to the present invention, the SABRE-identified polypeptides and respective antibodies have utility for use in a high-throughput method for *dual* (parallel) determination of orthopoxvirus immune response and orthopoxvirus infection. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Yet further aspects, the present invention provides an array of different orthopoxvirus (*e.g.*, monkeypox virus) peptide epitopes (*e.g.*, coupled to a solid phase).

In yet additional aspects, the present invention represents a surprising departure from the long-standing art-recognized dogma that particular immunological (*e.g.*, ELISA) assays have no utility for determination of *protective immunity* against orthopoxviruses, and particular embodiments provide rapid and reliable high-throughput methods for detecting protective immunity against orthopoxviruses (*e.g.*, for determination of protective immunity against smallpox virus, based on anti-vaccinia virus serum antibody levels. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Definitions

"Protective immunity" refers to the art-recognized protective immunity by a host, the immunity having been induced within the host by one or more prior vaccinations, or by one or more prior pathogen infections.

"Passive immunity" or "Immediate immunity" refers to the immunity conferred within a host, by passive antibody administration, wherein, passive antibody can theoretically confer protection regardless of the immune status of the host. Passive antibody administration can be used for post-exposure prophylaxis.

The term "SABRE" is an acronym for a novel method as disclosed herein for *s*ystematic *a*nalysis of *b*iologically *r*elevant *e*pitopes.

The term "epitope" refers herein, as is known in the art, to an antigenic determinant of a protein of polypeptide. An epitope could comprise 3 amino acids in a spacial conformation which is unique to the epitope. Generally an epitope consists of at least 5 such amino acids. An epitope of a polypeptide or protein antigen can be formed by contiguous or noncontinguous amino acid sequences of the antigen. A single viral protein, for example, may contain many epitopes. Additionally, a polypeptide fragment of a viral protein may contain multiple epitopes. The present invention encompasses epitopes and/or polypeptides recognized by antibodies of the present invention, along with conservative substitutions thereof, which are still recognized by the antibodies. Further truncation of these epitopes may be possible.

The term "Poxviridae" refers to viruses in the family Poxviridae, including poxviruses in the genera orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscipoxvirus and Yatapoxvirus which members include variola major and minor virus, monkeypox virus, camelpox virus, raccoonpox virus, ectromelia virus, sealpox virus, contagious ecthyma virus, canarypox virus, juncopox virus, pigeonpox virus, turkeypox virus, penguinpox virus, sheepox virus, goatpox, swinepox virus, buffalopox virus, cowpox virus, rabbit fibroma virus, myxoma virus, and molluscum contagiosum (genus Molluscipoxvirus) which is 59% identical and 77% similar to vaccinia (Altschul, S. F. et al. 1997, *Nucl. Acids Res.* 25, 3389-3402, fowlpox (genus Avipoxvirus), Yata-tumor like virus (Yatapoxvirus), among others (Fenner, Frank, Poxviruses, In "Virology" B. N. Fields et al., eds. Raves Press, Ltd. New York, 1990, pp. 2113-2133).

"Orthopoxviruses" refers, within the Poxviridae family, to a genus of closely related viruses that includes, but is not limited to, variola (smallpox), vaccinia, cowpox and monkeypox (all of which are known to infect humans), and also includes, but is not limited to camelpox, raccoonpox, skunkpox, volepox, ectromelia, and gerbilpox viruses.

"ELISA" refers to *e*nzyme-*l*inked *i*mmuno *s*orbent *a*ssays, as widely recognized in the art, and as described herein.

"Immunologic assay," as used herein refers to an art-recognized immunologic assay suitable to detect the formation of antigen:antibody complexes, including, but not limited to antibody capture assays, antigen capture assays, and two-antibody sandwich assays, ELISA, immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical and immuncytochemical techniques, Western analysis, agglutination and complement assays (see *e.g.*, *Basic and Clinical Immunology*, 217-262, Sites and Terr, eds., Appleton & Lange, Norwalk, CT, 1991 which is incorporated herein by reference). Preferred embodiments (*e.g.*, ELISA) of such assays are described herein below. According to the present invention, one or more of such immunoassays can be used to detect and/or quantitate antigens (*e.g.*, Harlow & Lane, Antibodies: A Laboratory Manual; Cold Spring Harbor Laboratory, New York 555-612, 1988, incorporated by reference herein).

The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow (lessen) pathogen (*e.g.*, viral) infection or associated conditions. Those in need of treatment include those already experiencing an infection, those prone to infection, and also those in which the potential infection is to be prevented.

"Antibodies," as used herein, refers to the art-recognized definition, and are described in more detail herein below.

"Neutralizing antibodies," as used herein, refers to the art-recognized definition.

"Cognate antigen," as used herein, refers to an antigen that is specifically bound by a cognate antibody, and "cognate antibody" refers to the antibody that specifically binds a cognate antigen.

"Parallel" or "dual" detection, as used herein refers to, detection, within a single sample, of both MPV infection and MPV-specific immune response. Preferably, detection of infection is contemporaneous with detection of a respective immune response to enable combined diagnostic use, but need not be simultaneous, and a plurality of immunologic assays and reagents. Preferably, parallel detection comprises use of at least one antigen, for detection of immune response, that is a cognate antigen of an antibody reagent used for detection of viral infection in the same sample.

"Orthpoxvirus proteins and polypeptides" as used herein encompasses both full-length orthopoxvirus proteins, as well as portions of such proteins, and includes 'peptides' and 'oligopeptides,' and additionally includes functional (*e.g.*, epitope-bearing, or antibody-binding) variants (including conservative amino acid sequence variants as described herein), fragments, muteins, derivatives and fusion proteins thereof.

"Vaccine," as used herein and in the art, refers to any type of biological agent in an administratable form capable of stimulating an immune response in an animal inoculated with the vaccine. For purposes of preferred embodiments of this invention, an inventive vaccine may comprise as the viral agent, one or more immunogenic (antigenic) components of the virus (*e.g.*, see TABLE 2 herein below for preferred antigens), and including polypeptide-based vaccines.

SABRE Technology (*S*ystematic *A*nalysis of *B*iologically *R*elevant *E*pitopes)

Preferred aspects of the present invention provide novel methods for systematic analysis of biologically relevant epitopes (SABRE), which enable rapidly and effective mapping/identification of biologically relevant (*e.g.*, immunodominant) peptide epitopes suitable for diagnostic and/or therapeutic applications.

Prior art methods for identification of biologically relevant peptide antigen/epitopes are "shotgun" approaches whereby a panel of uncharacterized antibodies, elicited by a particular antigen, are subsequently screened and tested to characterize the antibodies (*e.g.*, class, affinity, specificity, etc) to facilitate elucidation of the biological relevancy of the particular antigen/epitope. For example, a panel of antibodies generated against a particular viral antigen, might be screened and tested for the ability of the antibodies to neutralize virus and/or protect mice from viral challenge. Thus, such prior art approaches have great utility, once a biologically relevant antigen/epitope has been identified, but they do not provide an efficient method for initial selection of a biologically relevant antigen/epitope from among a large number of potentially relevant antigens and epitopes.

For example, U.S. Patent 6,620,412 to Hooper et al teaches a method for identification of potential targets for poxvirus therapeutics, comprising: initially generating a panel of 400 VACV-specific monoclonal antibodies (MAbs) in mice; and then characterizing the monoclonal antibodies by testing for their ability to neutralize virus and/or their ability to protect mice from challenge. Hooper et al used two challenge models, one that involves dissemination of the virus (in suckling mice), and another that involves a massive challenge dose (by intraperitoneal injection). Likewise, other prior art approaches are based on the same paradigm; namely, methods characterized by generation of antigen specific panel of antibodies, and subsequent characterization or properties and biological relevance.

The instant inventive systematic analysis of biologically relevant epitopes (SABRE) method provides a novel approach for rapidly and effectively mapping biologically relevant (*e.g.*, immunodominant) peptide epitopes suitable for diagnostic and/or therapeutic applications.

In preferred aspects, the method comprises: obtaining acute and/or convalescent serum from patients or naturally/experimentally infected animals who have recovered from a specific infectious disease or who are in the process of recovering from a specific infectious disease; obtaining specific polypeptides representing sub-regions of one or more proteins relevant to the infectious agent (*e.g.*, a set of polypeptides, based on genomic sequences and hydrophobicity plots) and using these polypeptides (*e.g.*, to create an array of polypeptides; to coat ELISA plates) for screening against positive and negative control sera; and identifying polypeptides/epitopes with high reactivity to positive control sera (*e.g.*, immunodominant epitopes) and low reactivity to negative control sera, thereby identifying biologically relevant epitopes.

Preferred proteins and polypeptides. Preferred proteins and polypeptides of the present invention are those of pathogenic viruses, such as orthopoxvirus proteins (*e.g.*, smallpox, vaccinia and monkeypox). In particular embodiments, they are of a strain of monkeypox virus. In other embodiments, they are a monkeypox virus (MPV) protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R. In particular embodiments, the proteins and polypeptides are selected from the group consisting of those listed in TABLE 2, herein below (SEQ ID NOS:1-29), and in TABLES 4, 5 and 6 (*e.g.*, SEQ ID NOS:30-44).

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2RR), 10 (N3R), 16 (B18R) and 20 (B21R), and epitope-bearing fragments of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2R), 10 (MPV N3R), 16 (MPV B18R) and 20 (MPV B21R).

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:2-5 (MPV D2L), 7-9 (MPV N2R), 11-15 (MPV N3R), 17-19 (MPV B18R) and 21-29, 30-44 (MPV B21R), and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:10 (MPV N3R) and 20 (MPV B21R), and epitope-bearing fragments of SEQ ID NOS:10 and 20.

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:11-15 (MPV N3R) and 21-29 (MPV B21R), and epitope bearing fragments of SEQ ID NOS: 11-15, 21-29 and 30-44.

In particular embodiments, the epitope comprises a sequence selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$) and 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

In particular embodiments, the epitope comprises a sequence selected from the group consisting of SEQ ID NO:31 and epitope-bearing fragments of SEQ ID NO:31.

Vaccines. In particular embodiments, the SABRE-identified polypeptides provide vaccines, based on the use of one or more SABRE-identified antigens in vaccine compositions. Such peptide-based vaccines are well known in the art, and may contain additional antigenic and adjuvant elements. Peptide-based vaccine are advantageous over traditional vaccines for several reasons: they are substantially safer; they have a relatively long shelf-life; they have the ability to target the immune response towards specific epitopes that are not suppressive nor hazardous for the host; and they offer the possibility of preparing multi-component and multi-pathogen vaccines.

The efficacy of inventive peptide-based vaccines are enhanced by adequate presentation of the epitopes to the immune system. Therefore, in preferred aspects, the orthopoxvirus (*e.g.*, monkeypox) antigens/epitopes are couple to, or are expressed (*e.g*, hydrid-gene expression) as part of, a carrier that may also offer an adjuvant function. Additional adjuvants may or may not be included in the immunization.

In particular aspects, immunizations are performed with one or more monkeypox virus (MPV) protein or polypeptide antigens selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R. In particular embodiments, the MPV protein or polypeptide is selected from the group

consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20. In particular embodiments, the MPV protein or polypeptide is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15, 21-29 and 30-44. In particular embodiments, the MPV protein or polypeptide is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27. In particular embodiments, the MPV protein or polypeptide is selected from the group consisting of SEQ ID NO:31 and epitope bearing fragments of SEQ ID NO:31.

Antibodies. In particular embodiments, SABRE-identified polypeptides have utility for developing respective antibodies (*e.g.*, monoclonal antibodies), and compositions comprising such antibodies.

Such antibodies and compositions have utility as novel *diagnostic* reagents for directly detecting the respective pathogen (*e.g.* for detecting orthopoxvirus infection, such as monkeypox infection). The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Diagnostic assays. Particular aspects of the present invention thus provide A high-throughput method for detecting monkeypox virus (MPV) infection, comprising: obtaining a test serum sample from a test subject; and detecting MPV in the sample using an immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

In particular embodiments, the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20. In particular embodiments, the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44. Preferably, the MPV protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10

and 20. In particular embodiments, the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:11-15, 21-29 and 30-44. In particular embodiments, the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27. In particular embodiments, the MPV polypeptide antigen is selected from the group consisting of SEQ ID NO:31 and epitope-bearing fragments of SEQ ID NO:31. In particular embodiments, the immunologic assay is selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, immunoelectrophoresis, immunochemical methods, Western analysis, antigen-capture assays, two-antibody sandwich assays, binder-ligand assays, agglutination assays, complement assays, and combinations thereof. In particular embodiments, the antibody is selected from the group consisting of a single-chain antibody, a monoclonal antibody, a chimeric antibody, a humanized antibody, and a Fab fragment. In particular embodiments, a plurality of antibodies, or eptitope-binding portions thereof, are used, in each case specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

Therapeutic agents. Additionally, because of the nature of the relevant specific binding interactions, antibodies and antibody-containing compositions of the present invention have *therapeutic utility* for treatment or prevention of orthopoxvirus (*e.g.*, smallpox, monkeypox and vaccinia) infections. The inventive antibodies and antibody compositions have utility for treating an infection, for alleviating symptoms of an infection, and/or to prevent pathogen infection. Preferably, the antibodies and antibody compositions are directed against monkeypox virus, or monkeypox virus proteins or polypeptides, and can be used to treat or prevent monkeypox virus infection by administration to subjects in need thereof.

Specifically, particular embodiments of the present invention provide an antibody directed against a monkeypox virus (MPV) protein or polypeptide antigen selected from the

group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

In particular embodiments, the antibody is a monoclonal antibody, or antigen-binding portion thereof. In particular embodiments, the monoclonal antibody, or antigen-binding portion thereof, is a single-chain antibody, chimeric antibody, humanized antibody or Fab fragment. Preferably, the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20. In particular embodiments, the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44. In particular embodiments, the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20. In particular embodiments, the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:11-15, 21-29 and 30-44. In particular embodiments, the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27. In particular embodiments, the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NO:31 and epitope-bearing fragments of SEQ ID NO:31.

Additional aspects provide a composition, comprising at least one of the above-described antibodies. Preferably, the composition comprises a N3R-specific monoclonal antibody, and a B21R-specific monoclonal antibody. Preferably, at least one of the antibodies forms specific immunocomplexes with monkeypox whole virions, or proteins or polypeptides associated with monkeypox virions.

Yet further aspects provide a *pharmaceutical* composition, comprising at least one of the above-described ntibodies of, along with a pharmaceutically acceptable diluent, carrier or excipient. Preferably, the composition is administered to a subject, whereby the composition prevents or inhibits monkeypox virus infection. In particular embodiments, the composition

is administered to a subject, whereby the composition ameliorates symptoms of monkeypox virus infection. In particular embodiments, at least one of the antibodies of the composition forms specific immunocomplexes with monkeypox whole virions, or proteins or polypeptides associated with monkeypox virions.

Yet further aspect provide a method of treating, or of preventing monkeypox virus infection, comprising administering to a subject in need thereof, a therapeutically effective amount of at least one of the above-described antibodies, or of a pharmaceutical composition comprising at least one of the antibodies. In particular embodiments, the immunoglobulin sequences are, or substantially are, human immunoglobulin sequences.

Detection of orthpoxvirus-specific immune response. In additional aspects, the present invention provides novel high-throughput assays for the detection of orthopoxvirus-specific *immune response*, based on measurement of orthopoxvirus-specific serum antibody levels. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations. Preferably, the orthopoxviruses include, but are not limited to smallpox, monkeypox and vaccinia viruses. EXAMPLE IV, herein below, describes the use of SABRE-identified polypeptides for detection of monkeypox virus-specific immune response (see also EXAMPLES V and VI).

Particular aspects provide a high-throughput method for detecting a monkeypox virus (MPV)-specific immune response, comprising: obtaining a test serum sample from a test subject; and detecting MPV-specific antibodies in the sample using an immunologic assay, based, at least in part, on use of at least one MPV protein or polypeptide selected from the group consisting of D2L, N2R, N3R, B18R, B21R, epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R; and combinations thereof.

In particular embodiments, the monkeypox virus (MPV) protein or polypeptide is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20. Preferably, the MPV polypeptide is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44. Preferably, the MPV protein or polypeptide is selected from the group consisting of SEQ ID NOS:10, 20 and

epitope-bearing fragments of SEQ ID NOS:10 and 20. Preferably, the MPV polypeptide is selected from the group consisting of SEQ ID NOS:11-15, 21-29, 30-44 and epitope bearing fragments of SEQ ID NOS:11-15, 21-29 and 30-44. In particular embodiments, the MPV polypeptide is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27. In particular embodiments, the MPV polypeptide is selected from the group consisting of SEQ ID NO:31 and epitope-bearing fragments of SEQ ID NO:31. In particular embodiments, the immunologic assay is selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof. In particular embodiments, a plurality of MPV proteins or polypeptides are used, in each case selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

In particular embodiments, detecting monkeypox virus (MPV)-specific antibodies in the sample further comprises determining an amount of MPV-specific antibodies in the sample, and the method further comprises determining, based at least in part on the amount of MPV-specific antibodies, a corresponding amount of MPV-neutralizing antibodies; thereby providing a determination of a level of *protective immunity* against MPV, based on a historic or contemporaneous correlation between amounts of MPV-neutralizing antibodies and levels of protective immunity against MPV. In particular embodiments, determining the amount of monkeypox virus (MPV)-neutralizing antibodies is by reference to a standard correlation between amounts of MPV-specific antibodies and amounts of MPV-neutralizing antibodies present in serum samples from previously vaccinated or infected individuals.

Dual, or parallel detection. Particularly preferred embodiments the SABRE-identified polypeptides and respective antibodies provide high-throughput *dual* (*parallel*) detection systems having utility for both direct detection of a particular pathogen, and for detecting immune response against the particular pathogen. Early during an infection, a pathogen will be present before a detectable immune response can be mounted. However, after an effective immune response is mounted (and/or disease symptoms arise), the pathogen sometimes becomes more difficult to detect, but the elicited immune response will remain for

an extended period. The inventive dual-detection SABRE reagents provide for: (i) direct and specific detection of the pathogen using extremely specific monoclonal antibody reagents (*i.e.*, antibodies specific the SABRE-identified immunodominant polypeptides); or (ii) specific detection of the immune response to the pathogen using the same unique pathogen-specific, SABRE-identified immunodominant polypeptides (*e.g.*, by using the polypeptides/antigens/epitopes to coat ELISA plates or using other immunoassay methods).

Significantly, a clinician has the highest likelihood of making a positive diagnosis, regardless of the stage of disease or infection, by using both detection methods simultaneously (or contemporaneously), so as to enable consideration of both detection results in the diagnosis with respect to a particular subject (or sample). The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

Particular embodiments provide a high-throughput method for parallel detection of both virus infection and immune response against the virus, comprising: obtaining a test serum sample from a test subject; detecting virus in the sample using a first immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for a viral protein or polypeptide antigen; and detecting viral-specific antibodies in the sample using a second immunologic assay, based, at least in part, on use of at least one of the viral proteins or polypeptides, wherein at least one of the proteins or polypeptides used for detecting virus-specific antibodies is the cognate antigen of one of the antibody reagents, or epitope binding portions thereof.

In particular embodiments, the immunologic assay is selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, immunoelectrophoresis, immunochemical methods, Western analysis, antigen-capture assays, antibody-capture assays, two-antibody sandwich assays, binder-ligand assays, agglutination assays, complement assays, and combinations thereof. In particular embodiments, a plurality of antibody reagents, or epitope-binding portions thereof, are used, and wherein a plurality of viral protein or polypeptide antigens are used. In particular embodiments, the plurality of antibody reagents, or epitope-binding portions thereof, and the plurality of viral protein or polypeptide antigens are cognate pairs. In particular embodiments, the virus is an

orthopoxvirus. In particular embodiments, the orthopoxvirus is selected from the group consisting of smallpox, vaccinia and monkeypox.

In particularly preferred embodiments, the invention provides a high-throughput method for parallel detection of both monkeypox virus (MPV) infection and MPV-specific immune response, comprising: obtaining a test serum sample from a test subject; detecting MPV in the sample using a first immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R; and detecting MPV-specific antibodies in the sample using a second immunologic assay, based, at least in part, on use of at least one of the MPV proteins or polypeptides, thereby providing for detection of both monkeypox virus (MPV) infection and MPV-specific immune response using the same serum sample.

In particular embodiments at least one of the proteins or polypeptides used for detecting MPV-specific antibodies is the cognate antigen of one of the antibody reagents, or epitope binding portions thereof. Preferably, the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20 (see also TABLE 2 herein below, and TABLES 4, 5 and 6). In particular embodiments, the first and second immunologic assay is, in each case, selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, immunoelectrophoresis, immunochemical methods, Western analysis, antigen-capture assays, antibody-capture assays, two-antibody sandwich assays, binder-ligand assays, agglutination assays, complement assays, and combinations thereof. In particular embodiments, the antibody reagent is selected from the group consisting of a single-chain antibody, a monoclonal antibody, a chimeric antibody, a humanized antibody, and a Fab fragment. In particular embodiments, a plurality of antibody reagents, or epitope-binding portions thereof, are used, and wherein a plurality of MPV protein or polypeptide antigens are used. In particular embodiments, the plurality of antibody

reagents, or epitope-binding portions thereof, and the plurality of MPV protein or polypeptide antigens are cognate pairs.

Therefore, the inventive SABRE platform provides benefits and applications at several levels, including the following four: *First*, the SABRE method yields the most immunodominant epitopes suitable for detecting an immune response against the pathogen (even at later stages of disease); *Second*, the SABRE method yields the most immunodominant epitopes suitable for development of diagnostic monoclonal antibodies; *Third* the SABRE provides for dual detection as described above, and *fourth*, the SABRE method yields the most immunodominant epitopes suitable for development of therapeutic monoclonal antibodies for treatment or prevention.

Arrays. Yet further embodiments provide an array of different Monkeypox virus proteins or polypeptides epitopes (oligopeptides) immobilized on a solid phase. The term "microarray" refers broadly to both 'polypeptide microarrays' and 'polypeptide chip(s),' and encompasses all art-recognized solid supports, and all art-recognized methods for synthesizing polypeptides on, or affixing polypeptides molecules thereto. The solid-phase surface may comprise, from among a variety of art-recognized materials, silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, gold or cellulose. However, nitrocellulose as well as plastics such as nylon, which can exist in the form of pellets or also as resin matrices, may also be used.

It is also anticipated that the oligopeptides, or particular sequences thereof, may constitute all or part of an "virtual array" wherein the oligopeptides, or particular sequences thereof, are used, for example, as 'specifiers' as part of, or in combination with a diverse population of unique labeled oligopeptides to analyze a complex mixture of analytes. In such methods, enough labels are generated so that each antibody in the complex mixture (*i.e.*, each analyte) can be uniquely bound by a unique label and thus be detected (*e.g.*, each label may be directly counted, resulting in a digital read-out of each molecular species in the mixture).

Preferred embodiments provide an array comprising a plurality of different monkeypox virus (MPV) proteins or polypeptides coupled to a solid phase, wherein the MPV

proteins or polypeptides are selected from the group consisting of of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

Preferably, the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20 (see also TABLE 2 herein below, and TABLES 4, 5 and 6). Preferably, the solid phase comprises a material selected from the group consisting of silicon, cellulose, glass, polystyrene, polyacrylamide, aluminum, steel, iron, copper, nickel, silver, gold and combinations thereof.

Protective Immunity Against Orthopoxviruses

Particular preferred aspects of the present invention provide novel methods for detection/measurement of *protective immunity* against specific orthopoxviruses (*e.g.*, smallpox, vaccinia and monkeypox).

There have been differing opinions with respect to what is required for full protective immunity against orthopoxviruses (Fenner et al., *The pathogenesis, immunology, and pathology of smallpox and vaccinia*; World Health Organization, Geneva, 1988). Two prospective studies (Mack et al., *Am J Trop Med Hyg*, 21:214-218, 1972; Sarkar et al., *Bull. World Health Organ.*, 52:307-311, 1975), along with a *study* comparing antibody titers to survival during active smallpox infection (Downie & McCarthy, *J. Hyg.*, 56:479-487, 1958), are consistent with a model in which high levels of neutralizing antibodies are at least associated with protective immunity against smallpox. Specifically, Mack et al. demonstrated that contacts of smallpox victims who had neutralizing titers of <1:32 were more susceptible to smallpox infection (3/15 (20%) contacts infected) than contacts with pre-existing antibody titers of ≥1:32 (0/127 (<1%) contacts infected). Likewise, Sarkar et al., in a smaller study, showed that 6/43 (14%) contacts with neutralizing titers of <1:20 contracted smallpox, whereas 0/13 contacts with titers ≥1:20 contracted the disease.

Significantly, however, these studies do not prove, and have not been regarded in the art as indicating a determinative role for neutralizing antibodies in protective immunity, since high levels of antiviral antibodies may have been passively associated with, for example,

higher underlying T cell memory. Moreover, and significantly, until the present invention, ELISA assays typically used for measurement of serum antibody levels, have been widely and dogmatically appreciated in the art as *not* having utility for determination of neutralizing antibodies and protective immunity.

The present invention represents a surprising departure from the long-standing art-recognized dogma that immunological (*e.g.*, ELISA) assays have no utility for determination of protective immunity against orthopoxviruses.

According to preferred aspects of the present invention, and consistent with the EXAMPLES disclosed herein below, serum antibody levels are a useful biomarker of protective immunity, regardless of whether protection is mediated by B cells, T cells, or a combination of both antiviral immune mechanisms.

According to the present invention, an orthopoxvirus-specific immunoassay (*e.g.*, ELISA) is used to detect or measure orthopoxvirus (*e.g.*, smallpox, vaccinia, monkeypox)-specific serum antibodies. The serum antibody levels are, in turn, correlated with a level of neutralizing antibodies, thereby providing a determination of a level of protective immunity against the orthopoxvirus, based on a historic or contemporaneous correlation between amounts of orthopoxvirus-neutralizing antibodies and levels of protective immunity against the orthopoxvirus.

In particular embodiments, the correlation between orthopoxvirus-specific serum antibodies and neutralizing antibodies is established by quantifying the levels of orthopoxvirus-specific neutralizing antibodies in appropriate serum samples (*e.g.*, vaccinated and unvaccinated individuals) using a corresponding orthopoxvirus plaque-reduction assay (*e.g.*, to determine the serum dilution at which 50% of the infectious virus is/was neutralized (NT_{50})).

The inventive assays are specific and sensitive, and have utility for reliably determining whether *protective immunity* exists against particular orthopoxviruses in particular individuals.

In preferred embodiments, specific anti-orthopoxvirus antibodies are detected by the inventive ELISA assays in collected serum samples as an indirect measurement of protective

immunity, and prior exposure. In particular embodiments, the orthopoxviruses include, but are not limited to smallpox, monkeypox and vaccinia viruses. Additionally, because some antibodies raised against vaccinia are cross reactive with other orthopoxviruses, including smallpox and monkeypox, the inventive system enables medical practitioners to determine the likelihood that a patient maintains protective immunity to multiple orthopoxviruses for years or decades following vaccination with vaccinia. The diagnostic assays are rapid, high-throughput and suitable for 'point-of-care' implementations.

In particular embodiments, the orthopoxviruses include, but are not limited to smallpox, monkeypox and vaccinia viruses.

Preferred aspects provide a high-throughput method for detecting *protective immunity* against smallpox virus, comprising: obtaining a test serum sample from a test subject previously vaccinated with a vaccinia-based vaccine; detecting an amount of vaccinia virus-specific antibodies in the sample using an immunologic assay; and determining, based at least in part on the amount of vaccinia virus-specific antibodies, a corresponding amount of vaccinia virus-neutralizing antibodies; thereby providing a determination of a level of protective immunity against smallpox virus, based on a historic correlation between amounts of vaccinia virus-neutralizing antibodies and protective immunity against small pox virus.

In particular embodiments, determining the amount of vaccinia virus-neutralizing antibodies is by reference to a historic or contemporaneous correlation between amounts of vaccinia virus-specific antibodies and amounts of vaccinia virus-neutralizing antibodies present in serum samples from individuals previously vaccinated with a vaccinia-based vaccine. In particular embodiments, the vaccinia virus-neutralizing antibodies comprise vaccinia intramolecular mature virus (IMV)-neutralizing antibodies In particular embodiments, the immunologic assay comprises an assay selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, immunoelectrophoresis, immunochemical methods, Western analysis, antigen-capture assays, antibody-capture assays, two-antibody sandwich assays, binder-ligand assays, agglutination assays, complement assays, and combinations thereof.

In particular embodiments, detecting an amount of vaccinia virus-specific antibodies in the sample using an immunologic assay, comprises forming immunocomplexes between the vaccinia virus-specific antibodies in the sample, and treated vaccinia virus, wherein the vaccinia virus has been treated with a peroxide agent prior to immunocomplex formation. In particular embodiments, the peroxide-treated vaccinia virus is immobilized on a surface prior to immumocomplex formation. In particular embodiments, treating of the vaccinia virus with a peroxide agent comprises treating with hydrogen peroxide. Preferably, during the treating, the hydrogen peroxide concentration is about 0.5% to about 10%, or about 1.0% to about 5%, or about 2% to about 4%, or about 3% (vol/vol).

Immunologic assays

According to the present invention, numerous art-recognized competitive and non-competitive protein binding immunoassays are used to detect and/or quantify antigens or antibodies (*e.g.*, Harlow & Lane, Antibodies: A Laboratory Manual; Cold Spring Harbor Laboratory, New York 555-612, 1988). Such immunoassays can be qualitative or and/or quantitative, and include, but are not limited to antibody capture assays, antigen capture assays, and two-antibody sandwich assays, immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays (*e.g.*, *Basic and Clinical Immunology*, 217-262, Sites and Terr, eds., Appleton & Lange, Norwalk, CT, 1991 which is incorporated herein by reference). Antibodies employed in such assays may be unlabeled, for example as used in agglutination tests, or labeled for use in a wide variety of assay methods. Labels that can be used include radionuclides, enzymes, fluorescers, chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes and the like for use in radioimmunoassay (RIA), enzyme immunoassays, *e.g.*, enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassays and the like.

Antibody capture assays comprise immobilizing an antigen on a solid support, and contacting the immobilized antigen with an antibody–containing solution, whereby antigen-specific antibody, if present, binds to the immobilized antigen. The antibodies can be labeled

or unlabeled. Antigen attachment to the solid support is typically non-covalent, but might in particular instances be covalent. After washing the support, antibody retained on the solid support is detected, or quantified by measuring the amount thereof. ELISA assays represent preferred embodiments of immunologic antibody capture assays as used herein. Competitive ELISA assays represent a preferred embodiment of antibody capture assay, wherein the antigen is bound to the solid support and two antibodies which bind the antigen (*e.g.*, serum from a orthopoxvirus vaccine, and a monoclonal antibody of the present invention) are allowed to compete for binding of the antigen. The amount of monoclonal antibody bound is measured, and a determination made as to whether the serum contains anti-orthopoxvirus antigen antibodies. Such ELISAs can be used to indicate immunity to known protective epitopes in a vaccinee following vaccination.

Antigen capture assays comprise immobilizing an antibody to a solid support, and contacting the immobilized antibody with an antigen–containing solution, whereby antibody-specific antigen, if present, binds to the immobilized antibody. The antigens can be labeled or unlabeled. Antibody attachment to the solid support is typically non-covalent, but might in particular instances be covalent. After washing the support, antigen retained on the solid support is detected, or quantified by measuring the amount thereof.

Two-antibody sandwich assays (*e.g.*, in the context of an antigen-capture assay) comprise initially immobilizing a first antigen-specific antibody on a solid support, followed by contacting the immobilized antibody with antigen-containing solution, washing the support, and subsequently detecting or quantifying the amount of bound antigen by contacting the immobilized antibody-antigen complexes with a second antigen-specific antibody, and measuring the amount of bound second antibody after washing.

Generally, immunoassays rely on labeled antigens, antibodies, or secondary reagents for detection. These proteins (antigens or antibodies) can be labeled with radioactive compounds, enzymes (*e.g.* peroxidase), biotin, or fluorochromes, etc. Enzyme-conjugated labels are particularly useful when radioactivity must be avoided, and provides for relatively rapid results. Biotin-coupled reagents are typically detected with labeled streptavidin. Streptavidin binds tightly and quickly to biotin and can be labeled with radioisotopes or

enzymes. Fluorochromes, provide a very sensitive method of detection. Antibodies useful in these assays include, but are not limited to, monoclonal antibodies, polyclonal antibodies, affinity-purified polyclonal antibodies, and antigen or epitope-binding fragments of any of these. Labeling of antibodies or fragments thereof can be accomplished using a variety of art-recognized techniques (*e.g.*, Kennedy et al., *Clin. Chim. Acta.*, 70:1-31, 1976; Schurs et al., *Clin. Chim Acta.*, 81:1-40, 1977; both incorporated by reference herein). Coupling techniques include, but are not limited to the glutaraldehyde, periodate method, dimaleimide and other methods.

ELISA. Enzyme-linked immunosorbent assay (ELISA) systems are widely recognized in the art, and are commonly used to detect antibodies in, for example, serum samples. For detection of antibodies in serum, a serum sample, or diluted serum sample, is applied to a surface (*e.g.* a well of a microtiter plate, preferably 'blocked' to reduce non-specific protein binding) having immobilized antigens (epitope(s)) thereon. Serum antibodies specific for the immobilized epitope(s) bind with high affinity to the immobilized epitope(s) on the plate, and are retained after standard washes, whereas non-specific antibodies do not bind with high affinity, and are removed after standard washes.

Specifically bound antibody is detected, for example, by using enzyme-coupled anti-immunoglobulins and a chromogen (*e.g.*, horseradish peroxidase-conjugated antibodies used in combination with hydrogen peroxide). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetirc or by visual means. Enzymes that can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

The detection can be accomplished by calorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished visually by comparison of the extent of enzymatic reaction with appropriate standards. Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect viral peptides peptides through the use of a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Inventive ELISA

In particular embodiments, a inventive vaccinia-specific ELISA, as disclosed herein below under EXAMPLE III, is preformed essentially as previously described using a

vaccinia-infected cell lysate (osmotic/freeze-thaw lysis) to coat 96–well flat-bottomed plates (Slifka & Ahmed, *J. Immunol. Methods,* 199:37-46, 1996)[48]. However, and significantly, in departure from prior art ELISA technology, nether heat nor a classic protein denaturant (*e.g.*, formaldehyde) is used to denature the vaccinia virus proteins prior to coating of the plates. Rather, in preferred embodiments for vaccinia-based ELISA, peroxide (*e.g.* hydrogen peroxide) is used to treat the vaccinia virus proteins (cell lysate) before coating the plates therewith. Preferably hydrogen peroxide is used to treat vaccinia virus at a concentration of at least 0.1%, at least 0.5%, at least 1.0%, and least 2 % at least 3%, at least 5%, or at least 10%, but less than about 20% or 30%. Preferably the hydrogen peroxide concentration is in a range of about 0.5% to about 10%, or about 1.0% to about 5%, or about 2% to about 4%, or about 3%. Preferably the peroxide concentration is about 3%.

Significantly, in preferred aspects for vaccinia virus-based ELISA, substitution of peroxide (*e.g.*, hydrogen peroxide) in place of heat or classic protein denaturants (*e.g.*, formaldehyde) enables detection of anti-vaccinia serum antibody levels that are correlatable with a level of neutralizing antibodies, thereby providing a determination of a level of protective immunity against an orthopoxvirus (or cross-reactive orthopoxvirus), based on a historic or contemporaneous correlation between amounts of orthopoxvirus-neutralizing antibodies and levels of protective immunity against the orthopoxvirus.

Neutralization Assays

Neutralization assays, as disclosed herein (see EXAMPLE III below), were performed following an optimized protocol similar to that previously described (Mack et al., *Am. J. Trop. Med. Hyg.,* 21:214-218, 1972; Cutchins et al., *J. Immunol.,* 85:275-283, 1960)[8,50].

Generation and Production of Antibodies

Polyclonal or monoclonal antibodies to orthopoxvirus proteins and polypeptides or to epitope-bearing fragments thereof can be made for therapeutic, or diagnostic (*e.g.*, immunoassays) use by any of a number of methods known in the art. By epitope reference is made to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids

in a spatial conformation which is unique to the epitope (methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2 dimensional nuclear magnetic resonance). Generally an epitope consists of at least 5 such amino acids. The present invention encompasses epitopes and/or polypeptides recognized by antibodies of the present invention, along with conservative substitutions thereof, which are still recognized by the antibodies.

One approach for preparing antibodies to a protein is the selection and preparation of an amino acid sequence of all or part of the protein, chemically synthesizing the sequence and injecting it into an appropriate animal, usually a rabbit or a mouse.

Oligopeptides can be selected as candidates for the production of an antibody to orthopoxvirus proteins or polypeptides based upon the oligopeptides lying in hydrophilic regions, which are thus likely to be exposed in the mature protein.

Alternatively, proteins and polypeptides can be selected by the inventive SABRE method disclosed herein. Additionally, a combination of selection methods can be used.

Preferred proteins and polypeptides of the present invention are those of pathogenic viruses, such as orthopoxvirus proteins (*e.g.*, smallpox, vaccinia and monkeypox). Preferably, they are of a strain of monkeypox virus. Preferably, they are a monkeypox virus (MPV) protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R. Preferably, the proteins and polypeptides are selected from the group consisting of those listed in TABLEs 2, 4, 5 and 6herein below (SEQ ID NOS:1-29 and 30-44).

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2RR), 10 (N3R), 16 (B18R) and 20 (B21R), and epitope-bearing fragments of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2R), 10 (MPV N3R), 16 (MPV B18R) and 20 (MPV B21R).

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:2-5 (MPV

D2L), 7-9 (MPV N2R), 11-15 (MPV N3R), 17-19 (MPV B18R) and 21-29 (MPV B21R), and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:10 (MPV N3R) and 20 (MPV B21R), and epitope-bearing fragments of SEQ ID NOS:10 and 20.

In particular embodiments, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:11-15 (MPV N3R) and 21-29 (MPV B21R), and epitope bearing fragments of SEQ ID NOS: 11-15, 21-29 and 30-44.

In particular embodiments, the epitope comprises a sequence selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$) and 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27. Preferably, the epitope comprises a sequence selected from the group consisting of SEQ ID NO: 31 and epitope-bearing fragments of SEQ ID NO:31.

Preferred proteins and oligopeptides of the present invention are shown in TABLES 2, 4, 5 and 6 (under EXAMPLE IV, V and VI herein below).

Methods for preparation of the orthopoxvirus proteins or polypeptides, or of an epitope thereof include, but are not limited to chemical synthesis, recombinant DNA techniques or isolation from biological samples. Chemical synthesis of a peptide can be performed, for example, by the classical Merrifeld method of solid phase peptide synthesis (Merrifeld, *J. Am. Chem. Soc. 85*:2149, 1963 which is incorporated by reference) or the FMOC strategy on a Rapid Automated Multiple Peptide Synthesis system (E. I. du Pont de Nemours Company, Wilmington, DE) (Caprino and Han, *J Org Chem 37*:3404, 1972 which is incorporated by reference).

Polyclonal antibodies can be prepared by immunizing rabbits or other animals by injecting antigen followed by subsequent boosts at appropriate intervals. The animals are bled and sera assayed against purified orthopoxvirus proteins or polypeptides usually by ELISA or by bioassay based upon the ability to block the action of orthopoxvirus proteins or polypeptides. When using avian species, *e.g.*, chicken, turkey and the like, the antibody can

be isolated from the yolk of the egg. Monoclonal antibodies can be prepared after the method of Milstein and Kohler by fusing splenocytes from immunized mice with continuously replicating tumor cells such as myeloma or lymphoma cells. (Milstein and Kohler, *Nature 256*:495-497, 1975; Gulfre and Milstein, *Methods in Enzymology: Immunochemical Techniques 73*:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). The hybridoma cells so formed are then cloned by limiting dilution methods and supernates assayed for antibody production by ELISA, RIA or bioassay.

The unique ability of antibodies to recognize and specifically bind to target proteins provides an approach for treating infectious disease. Thus, another aspect of the present invention provides for a method for preventing or treating diseases involving treatment of a subject with specific antibodies to orthopoxvirus proteins or polypeptides.

Specific antibodies, either polyclonal or monoclonal, to the orthopoxvirus proteins or polypeptides can be produced by any suitable method known in the art as discussed above. For example, murine or human monoclonal antibodies can be produced by hybridoma technology or, alternatively, the orthopoxvirus proteins or polypeptides, or an immunologically active fragment thereof, or an anti-idiotypic antibody, or fragment thereof can be administered to an animal to elicit the production of antibodies capable of recognizing and binding to the orthopoxvirus proteins or polypeptides. Such antibodies can be from any class of antibodies including, but not limited to IgG, IgA, IgM, IgD, and IgE or in the case of avian species, IgY and from any subclass of antibodies.

The present invention further provides for methods to detect the presence of the orthopoxvirus proteins or polypeptides in a sample obtained from a patient. As discussed above under "Immunologic Assays," any method known in the art for detecting proteins can be used. Such methods include, but are not limited to immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays. (for example, see *Basic and Clinical Immunology*, 217-262, Sites and Terr, eds., Appleton & Lange, Norwalk, CT, 1991 which is incorporated by reference). Preferred are ELISA methods, including reacting antibodies with an epitope or epitopes of the orthopoxvirus proteins or polypeptides.

As provided herein, the compositions and methods for diagnosis/detection of viral infection, or the therapeutic methods of treatment or prevention provided herein, may utilize one or more antibodies used singularly, or in combination with other therapeutics to achieve the desired effects. Antibodies according to the present invention may be isolated from an animal producing the antibody as a result of either direct contact with an environmental antigen or immunization with the antigen. Alternatively, antibodies may be produced by recombinant DNA methodology using one of the antibody expression systems well known in the art (*see, e.g.*, Harlow & Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988). Such antibodies may include recombinant IgGs, chimeric fusion proteins having immunoglobulin derived sequences or "humanized" antibodies that may all be used according to the present inventive aspects. In addition to intact, full-length molecules, the term antibody also refers to fragments thereof (*e.g.*, scFv, Fv, Fd, Fab, Fab' and $F(ab)'_2$ fragments), or multimers or aggregates of intact molecules and/or fragments that bind to the inventive antigens (proteins/polypeptides/epitopes). These antibody fragments bind antigen and may be derivatized to exhibit structural features that facilitate clearance and uptake (*e.g.*, by incorporation of galactose residues).

In particular embodiments antibodies are monoclonal antibodies, prepared essentially as described in Halenbeck et al. U.S. Patent Number 5,491,065 (1997), incorporated herein by reference.

Additional embodiments comprise humanized monoclonal antibodies. The phrase "humanized antibody" refers to an antibody initially derived from a non-human antibody, typically a mouse monoclonal antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans. The phrase "chimeric antibody," as used herein, refers to an antibody containing sequence derived from two different antibodies (*see, e.g.*, U.S. Patent Number 4,816,567) which typically originate from different species. Most typically, chimeric antibodies comprise human and murine antibody fragments, generally human constant and mouse variable regions.

Because humanized antibodies are less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of anaphylaxis. Thus, these antibodies may be preferred in therapeutic applications that involve *in vivo* administration to a human.

Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as "humanizing"), or, alternatively, (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"). In the present invention, humanized antibodies will include both "humanized" and "veneered" antibodies. These methods are disclosed in, for example, Jones et al., *Nature* 321:522-525, 1986; Morrison et al., *Proc. Natl. Acad. Sci., U.S.A.*, 81:6851-6855, 1984; Morrison and Oi, *Adv. Immunol.*, 44:65-92, 1988; Verhoeyer et al., *Science* 239:1534-1536, 1988; Padlan, *Molec. Immun.* 28:489-498, 1991; Padlan, *Molec. Immunol.* 31(3):169-217, 1994; and Kettleborough, C.A. et al., *Protein Eng.* 4(7):773-83, 1991, each of which is incorporated herein by reference.

The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site (*see, e.g.*, Chothia et al., *J. Mol. Biol.* 196:901-917, 1987; Kabat et al., U.S. Dept. of Health and Human Services NIH Publication No. 91-3242, 1991). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In the present invention, mouse constant regions are substituted by human constant regions. The constant regions of the subject humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu.

One method of humanizing antibodies comprises aligning the non-human heavy and light chain sequences to human heavy and light chain sequences, selecting and replacing the non-human framework with a human framework based on such alignment, molecular modeling to predict the conformation of the humanized sequence and comparing to the

conformation of the parent antibody. This process is followed by repeated back mutation of residues in the CDR region which disturb the structure of the CDRs until the predicted conformation of the humanized sequence model closely approximates the conformation of the non-human CDRs of the parent non-human antibody. Such humanized antibodies may be further derivatized to facilitate uptake and clearance (*e.g.*, via Ashwell receptors) (*see, e.g.*, U.S. Patent Numbers 5,530,101 and 5,585,089, both incorporated herein by reference).

Humanized antibodies to the inventive proteins can also be produced using transgenic animals that are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin encoding loci are substituted or inactivated. WO 96/30498 discloses the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or variable region to form a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent Number 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

Using a transgenic animal described above, an immune response can be produced to a selected antigenic molecule, and antibody producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735; disclosing monoclonal antibodies against a variety of antigenic molecules including IL-6, IL-8, TNFa, human CD4, L-selectin, gp39, and tetanus toxin. The monoclonal antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein or pathogenic agent (*e.g.*, virus). WO 96/3373 discloses that monoclonal antibodies against IL-

8, derived from immune cells of transgenic mice immunized with IL-8, blocked IL-8 induced functions of neutrophils. Human monoclonal antibodies with specificity for the antigen used to immunize transgenic animals are also disclosed in WO 96/34096. The antibodies of the present invention are said to be immuospecific, or specifically binding, if they bind to the viral antigen (protein/polypeptide/epitope) with a K_a of greater than or equal to about 10^4 M^{-1}, preferably of greater than or equal to about 10^5 M^{-1}, more preferably of greater than or equal to about 10^6 M^{-1}, and still more preferably of greater than or equal to about 10^7 M^{-1}. Such affinities may be readily determined using conventional techniques, such as by equilibrium dialysis; by using the BIAcore 2000 instrument, using general procedures outlined by the manufacturer; by radioimmunoassay using ^{125}I-labeled proteins; or by another method known to the skilled artisan. The affinity data may be analyzed, for example, by the method of Scatchard et al., *Ann N.Y. Acad. Sci.*, 51:660, 1949. Thus, it will be apparent that preferred antibodies will exhibit a high degree of specificity for the viral antigen of interest, and will bind with substantially lower affinity to other molecules.

Preferably the anti-pathogenic antibodies of the present invention are monoclonal antibodies. More preferably, the antibodies are humanized monoclonal antibodies.

The present invention is further illustrated by reference to the EXAMPLES below. However, it should be noted that these EXAMPLES, like the embodiments described above, are illustrative and are not to be construed as restricting the enabled scope of the invention in any way.

EXAMPLE I

(CD4$^+$ T cell-mediated immune responses were evaluated in volunteers examined at 1 month to 75 years post-vaccination, and significant CD4$^+$ T cell responses were detected as late as 75 years post-immunization)

Quantification of virus-specific CD4$^+$ T cell responses. Very little is known about the duration of vaccinia-specific T cell responses or what proportion of vaccinated individuals will maintain detectable levels of CD4$^+$ and/or CD8$^+$ T cell memory. To shed light on this fundamental question, the maintenance of virus-specific immunity after smallpox vaccination

was analyzed by conducting a non-randomized, cross-sectional analysis of $CD4^+$ T cell-mediated immune responses in volunteers examined at 1 month to 75 years post-vaccination. Although the frequency of virus-specific $CD4^+$ T cells waned slowly over time, T cell responses in most subjects remained at levels within 1–2 orders of magnitude of those achieved at ≤7 years post-vaccination and could be detected as late as 75 years post-immunization.

$CD4^+$ T cell responses were quantified using a highly optimized intracellular cytokine staining (ICCS) protocol that detects vaccinia-specific T cells by virtue of their ability to produce potent antiviral factors including IFN-γ and TNF-α following exposure to vaccinia directly ex vivo (FIGURE 1A). FIGURES 1A and 1B show the levels of Virus-specific $CD4^+$ T cell memory following smallpox vaccination.

FIGURE 1A illustrates a representative flow cytometry dotplot gated on $CD4^+CD8^-$ T cells showing the number of IFN-γ^+ TNF-α^+ events calculated per million $CD4^+$ T cells (+Vaccinia) after background subtraction (–Vaccinia) in PBMC samples from an unvaccinated volunteer, or from volunteers analyzed at 1 or 61 years post-vaccination. After background subtraction (–Vaccinia), IFN-γ^+TNF-α^+ $CD4^+$ T cells were below detection in the representative unvaccinated control (<$10/10^6$ $CD4^+$ T cells), but readily observed at 1-year post-vaccination ($586/10^6$ $CD4^+$ T cells) as well as at 61 years post-vaccination ($56/10^6$ $CD4^+$ T cells). In both of these latter cases, the number of IFN-γ^+TNF-α^+ events in the vaccinia-stimulated samples (+Vaccinia) was more than 10–fold higher than those observed in the unstimulated (–Vaccinia) controls cultured in parallel. Moreover, in 7 consecutive experiments, samples from the same volunteer at 1 year post-vaccination averaged 622±125 IFN-γ^+TNF-α^+ $CD4^+$ T cells per million total $CD4^+$ T cells, indicating that this is a highly reproducible assay.

Significantly, approximately 90% of IFN-γ^+ vaccinia-specific $CD4^+$ T cells co-expressed TNF-α, indicating that they maintained a "memory phenotype" of dual cytokine expression (Slifka & Whitton, *J. Immunol.*, 164:208-216, 2000)[11]. Subpopulations of IFN-γ^+TNF-α^- and IFN-γ^-TNF-α^+ T cells were also observed in some, but not all, individuals (*e.g.*, FIGURE 1A). The most conservative estimates obtained by enumeration of functional

T cells capable of dual IFN-γ and TNF-α production were relied on for quantification of the duration of $CD4^+$ T cell memory.

FIGURE 1B shows the quantification of virus-specific $CD4^+$ T cells as a function of time post-vaccination. Following vaccination or revaccination, virus-specific $CD4^+$ T cells were detected in 18/18 vaccinees at 27–34 d post-immunization (average = $900/10^6$ $CD4^+$ T cells) and then declined slowly with a half-life of 8 to 12 years (FIGURE 1B and TABLE 1).

TABLE 1, below, shows the estimated survival of virus-specific T cell memory following smallpox vaccination. Interestingly, although multiple vaccinations are believed to provide maximum long-term protection (Nyerges et al., *Acta Microbiol. Acad. Sci., Hung.* 19:63-68, 1972; el-Ad et al., *J. Infect. Dis.*, 161:446-448, 1990)[12,13], repeated exposure to vaccinia did not greatly alter the magnitude (FIGURE 1B), or the half-life of T cell memory (TABLE 1).

Significantly, although the frequency of virus-specific $CD4^+$ T cells waned slowly over time, T cell responses in most subjects remained at levels within 1–2 orders of magnitude of those achieved at ≤7 years post-vaccination and could be detected as late as 75 years post-immunization.

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TABLE 1. Estimated survival of virus-specific T cell memory following smallpox vaccination.

	% of volunteers with $CD4^+$ T cell memory[a]			
# Vaccinations	20–30 years[c]	31–50 years	51–75 years	$T_{1/2}$ of $CD4^+$ T cells[b]
1	100% (16/16)	89% (70/79)	52% (23/44)	10.6 (0–17)[d]
2	83% (10/12)	78% (29/37)	57% (4/7)	8.3 (0–14.1)
3–14	82% (23/28)	91% (29/32)	N.D.[e]	12.4 (0–20.5)
	% of volunteers with $CD8^+$ T cell memory			

vaccinations	20–30 years	31–50 years	51–75 years	$T_{1/2}$ of $CD8^+$ T cells
1	50% (8/16)	49% (39/79)	50% (22/44)	15.5 (0–27.1)
2	42% (5/12)	38% (14/37)	57% (4/7)	8.1 (0–16.9)
3–14	46% (13/28)	50% (16/32)	N.D.	9.0 (0–18.1)

[a]Percentage of volunteers with vaccinia-specific T cell memory was based on the proportion of immunized participants with >10 IFN-γ^+TNF-α^+ T cells/10^6 $CD4^+$ or $CD8^+$ T cells, respectively. This cut-off point provided 100% sensitivity at 1-month post-vaccination/revaccination and 92–96% specificity, based on the vaccinia-induced IFN-γ response in T cells from unvaccinated volunteers.

[b]Estimated $T_{1/2}$ in years was based on linear regression analysis using the data from FIGURES 1 and 2.

[c]Years after the last smallpox vaccination.

[d]95% Confidence Intervals.

[e]N.D., Not Determined.

EXAMPLE II

($CD8^+$ T cell-mediated immune responses were evaluated in volunteers examined at 1 month to 75 years post-vaccination, and significant $CD8^+$ T cell responses were detected as late as 75 years post-immunization)

Quantification of virus-specific $CD8^+$ T cell responses. The maintenance of virus-specific immunity after smallpox vaccination was also analyzed by conducting a non-randomized, cross-sectional analysis of antiviral antibody and $CD8^+$ T cell-mediated immune responses in volunteers examined at 1 month to 75 years post-vaccination. Robust $CD8^+$ T cell responses were identified (FIGURE 2B), and similar to $CD4^+$ T cells (FIGURE 1B), $CD8^+$ T cells declined slowly with a half-life of 8 to 15 years (TABLE 1).

Antiviral $CD8^+$ T cell responses were quantified by ICCS following direct *ex vivo* stimulation with vaccinia-infected cells (FIGURE 2A).

Figure 2 shows the levels of virus-specific $CD8^+$ T cell memory following smallpox vaccination. FIGURE 2A shows a representative flow cytometry dotplot gated on $CD8^+CD4^-$ T cells showing the number of IFN-γ^+ TNF-α^+ events calculated per million $CD8^+$ T cells (+Vaccinia) after background subtraction (–Vaccinia) in PBMC samples from an unvaccinated volunteer, or from volunteers analyzed at 1 or 61 years post-vaccination.

FIGURE 2B shows the quantitation of virus-specific $CD8^+$ T cells as a function of time post-vaccination.

Although a recent study has identified two HLA-A*0201-restricted T cell epitopes (Terajima et al., *J. Exp. Med.*, 197:927-932, 2003)[14], these epitopes measure only a subpopulation of the total T cell response (supra)[14] and therefore live virus was used to stimulate T cells in this cross-sectional study so that the global antiviral $CD8^+$ T cell response could be identified, irrespective of the HLA type of the donor (Speller & Warren, *J. Immunol. Methods*, 262:167-180, 2002)[15], and to allow side-by-side comparisons with $CD4^+$ T cell responses (for which no epitopes have yet been mapped).

The majority of IFN-γ^+CD8^+ T cells co-expressed TNF-α and again we used dual cytokine production as the functional criteria for quantitating virus-specific T cell memory. Samples from one volunteer (1 year post-vaccination) averaged 2,215 ± 325 IFN-γ^+TNF-α^+ $CD8^+$ T cells per million $CD8^+$ T cells in 7 consecutive experiments. At 27–34 d post-vaccination or revaccination, robust $CD8^+$ T cell responses (average = $870/10^6$ $CD8^+$ T cells) were identified in 18/18 vaccinees (FIGURE 2B). Similar to $CD4^+$ T cells (FIGURE 1B), $CD8^+$ T cells declined slowly with a half-life of 8 to 15 years (TABLE 1). Comparison of $CD8^+$ T cell levels following booster vaccination did not reveal any substantial improvements in long-term T cell memory above that observed following a single vaccination (FIGURE 2B and TABLE 1).

Direct comparisons between virus-specific $CD4^+$ and $CD8^+$ T cell levels within individual vaccinees revealed dynamic and independently regulated changes in T cell memory over time (FIGURE 3).

Figure 3 shows the relationship between vaccinia-specific $CD4^+$ and $CD8^+$ T cell memory over time. Comparisons were made between the number of antiviral $CD4^+$ and $CD8^+$ T cells from the same individual. FIGURE 3A shows 1 month to 7 years post-vaccination, whereas FIGURE 4B shows 14 to 40 years post-vaccination, and FIGURE 4C shows 41 to 75 years post-vaccination.

At early time points ranging from 27-days to 7-years post-vaccination, nearly all of the volunteers possessed strong $CD4^+$ and $CD8^+$ T cell responses (FIGURE 3A).

At later time points, examined between 14–40 years post-vaccination (FIGURE 3B) or 41–75 years post-vaccination (FIGURE 3C), many individuals still maintained both $CD4^+$ and $CD8^+$ T cell memory (albeit at lower levels than earlier time points observed in FIGURE 3A), but other individuals preferentially lost $CD8^+$ T cell memory while leaving the antiviral $CD4^+$ T cell compartment intact.

In rare cases, $CD8^+$ T cell responses remained elevated while $CD4^+$ T cell responses dropped to below detection. Further studies will be necessary to determine why virus-specific $CD8^+$ T cells, or in some cases, $CD4^+$ T cells are disproportionably lost over prolonged periods of time, but the overall shift in T cell memory appears to reflect the antiviral $CD4^+$ and $CD8^+$ T cell survival rates (TABLE 1).

EXAMPLE III

(The duration of antiviral antibodies were examined in volunteers examined at 1 month to 75 years post-vaccination, and vaccinia-specific serum antibody levels were found to be remarkably stable between 1 year to 75 years post-vaccination)

Duration of antiviral antibody production. The maintenance of virus-specific immunity after smallpox vaccination was analyzed by conducting a non-randomized, cross-sectional analysis of antiviral antibodies in volunteers examined at 1 month to 75 years post-vaccination. In striking contrast to vaccinia-specific T cell memory which declined steadily over time (FIGURES 1 and 2), vaccinia-specific serum antibody levels were remarkably stable between 1 year to 75 years post-vaccination (FIGURE 4).

Vaccinia-specific neutralizing antibody titers have been the cardinal feature used to estimate the level of immunity afforded by smallpox vaccination (Fenner et al. in *The pathogenesis, immunology, and pathology of smallpox and vaccinia,* World Health Organization, Geneva, 1988; Downie & McCarthy, *J. Hyg.,* 56:479-487, 1958; McCarthy & Downie, *J. Hyg.,* 56:466-478, 1958; Stienlauf et al., *Vaccine,* 17:201-204, 1999; CDC, *MMWR,* 50:1-25, 2001; Frey et al., *JAMA,* 289:3295-3299, 2003)[7,10,16-19]. To examine this issue in more detail, a sensitive, reproducible, and validated vaccinia-specific ELISA was developed for high-throughput analysis of humoral immunity following smallpox vaccination.

Inventive ELISA assay. The ELISA for detection of anti-vaccinia virus antibodies was preformed essentially as previously described using a vaccinia-infected cell lysate (osmotic/freeze-thaw lysis) to coat 96–well flat-bottomed plates (Slifka & Ahmed, *J. Immunol. Methods,* 199:37-46, 1996)[48]. Significantly however, and in departure from prior art ELISA technology, nether heat nor a classic protein denaturant (*e.g.*, formaldehyde) was used to denature the vaccinia virus proteins prior to coating of the plates. Rather, peroxide (*e.g.* hydrogen peroxide) was used to treat the vaccinia virus proteins (cell lysate) before coating the plates therewith. Preferably peroxide is used to treat vaccinia virus-containing solutions at a concentration of at least 0.5%, at least 1.0%, and least 2 % at least 3%, at least 5%, or at least 10%. Preferably the peroxide concentration is in a range of about 0.5% to about 10%, or about 1.0% to about 5%, or about 2% to about 4%, or about 3%. Preferably the peroxide concentration is about 3%. The data in this exemplary analysis was obtained the treating the vaccinia virus in 3% hydrogen peroxide prior to coating plates therewith.

According to the present invention, substitution of peroxide (*e.g.*, hydrogen peroxide) in place of heat or classic protein denaturants (*e.g.*, formaldehyde) enables detection of anti-vaccinia serum antibody levels that are correlatable with a level of neutralizing antibodies, thereby providing a determination of a level of protective immunity against an orthopoxvirus, based on a historic or contemporaneous correlation between amounts of orthopoxvirus-neutralizing antibodies and levels of protective immunity against the orthopoxvirus.

Serial 3–fold dilutions of serum were incubated on pre-blocked ELISA plates for 1 h, washed, incubated with mouse anti-human IgG-HRP (clone G18-145, Pharmingen), washed, detection reagents added, and samples analyzed on a VERSA$_{max}$™ ELISA plate reader (Molecular Devices). The WHO International Standard for anti-smallpox serum (Anderson & Skegg, *Bull. World Health Organ.*, 42:515-523, 1970)[49] was used to calibrate antiviral IgG measured by ELISA and an internal positive control was included on every plate in order to normalize ELISA values between plates and between assays performed on different days. Antibody titers were determined by logarithmic transformation of the linear portion of the curve with 0.1 OD units used as the endpoint and conversion performed on final values.

Figure 4 shows long-lived antiviral antibody responses induced by smallpox vaccination. FIGURE 4A shows the quantitation of vaccinia-specific antibody responses by ELISA. FIGURE 4B shows the levels of vaccinia-specific antibody titers (1 to 75 years post-vaccination) compared to the total number of vaccinations received. FIGURE 4C shows the correlation between virus-specific antibody titers determined by ELISA and by neutralizing assays was determined by linear regression analysis after plotting the log values obtained from serum samples of volunteers vaccinated one or two times against smallpox. The slope of the line was defined as Log NT_{50} = 0.056+0.487Log ELISA. R^2 = 0.450, P < 0.0001. The relationship between virus-specific $CD4^+$ (closed symbols) or $CD8^+$ (open symbols) T cells (per million $CD4^+$ or $CD8^+$ T cells, respectively) with virus-specific antibody titers was determined at 1 month to 7 years post-vaccination (CD4; P = 0.67, CD8; P = 0.39) as shown FIGURE 4D 14 years to 40 years post-vaccination (CD4; P = 0.72, CD8; P = 0.89), as shown in FIGURE 4E, or 41 years to 75 years post-vaccination (CD4; P = 0.77, CD8; P = 0.06*) as shown in FIGURE 4F.

Using 100 ELISA Units (EU) as the lowest titer considered to be positive, 100% specificity (0/26 unvaccinated controls scored ≥100 EU) and 98% sensitivity (288/293 samples from volunteers vaccinated against smallpox scored ≥100 EU) was observed. One representative positive control (scoring 644 EU) was repeated >40 times and varied by <12% within a single assay and varied by <18% between assays, with 0% false-negative results. Likewise, a representative negative control sample from an unvaccinated volunteer was repeated in >40 assays and in each case scored <50 EU, with 0% false-positive results.

In striking contrast to vaccinia-specific T cell memory which declined steadily over time (FIGURES 1 and 2), vaccinia-specific serum antibody levels were remarkably stable between 1 year to 75 years post-vaccination and we were unable to determine a half-life of antibody decay. Comparison of antiviral antibody titers elicited by one or more vaccinations revealed a very small (<2–fold), but statistically significant increase in the mean level of antibody produced after 2 vaccinations in comparison with only 1 vaccination (P=0.02, FIGURE 4B). However, additional vaccinations ranging from 3–5 or as many as 6–14 immunizations did not result in any further increases in long-term antibody production. This

indicates that booster vaccination may increase a previously suboptimal antibody response, but is unlikely to induce prolonged synthesis of higher antibody levels above a certain threshold level.

ELISA assays do not directly measure levels of neutralizing antibodies and must therefore be validated side-by-side with neutralizing assays in order for them to be useful as a means of quantitating biologically relevant antibody levels. By performing neutralizing assays in essentially the same manner as that described in previous studies in which an experimental value for protective immunity was defined ($NT_{50}\geq1:32$) (Mack et al., *supra*)[8], the data was directly related to historical findings that can not be repeated now that natural smallpox is extinct.

Specifically, several 3–fold dilutions (beginning at 1:4 or 1:12) of heat-inactivated serum were incubated with vaccinia (~100 plaque forming units) for 2 h at 37 °C before incubating the virus with Vero cells for 1-h, overlaying with 0.5% agarose and incubating for 3.5 days to allow plaque formation. Cells were fixed with 75% methanol, 25% acetic acid, and after removing the agarose, plaques were visualized by staining with 0.1% crystal violet in PBS containing 0.2% formaldehyde. The neutralization titer (NT_{50}) was defined as the reciprocal of the serum dilution required for 50% reduction in vaccinia plaques. Logarithmic transformation of the data was used to calculate the titer and conversion was done on final values, excluding those in which ≥85% neutralization occurred. Antibodies against the extracellular enveloped virus (EEV) are highly protective *in vivo* (Galmiche et al., *Virology*, 254:71-80, 1999)[30] but so are antibodies against IMV (Czerny & Mahnel, *J. Gen. Virol.*, 71:2341-2352, 1990; Ramirez et al., *J. Genl. Virol.*, 83:1059-1067, 2002)[29,31] and we chose IMV for our neutralization studies because there is a precedent for protective immunity against smallpox if the individuals have pre-existing neutralizing antibody titers (against IMV) that are ≥1:20 (Sarkar et al., *Bull. World Health Organ.*, 52:307-311, 1975)[9] or ≥1:32 (Mack et al., *Am. J. Trop. Med. Hyg.*, 21:214-218, 1972)[8]. EEV would be unsuitable for these assays because we would not be able to compare our results to these historical values.

Significantly, a direct linear relationship ($P<0.0001$) was observed between neutralizing titers and the levels of virus-specific antibodies quantitated by the inventive

ELISA (FIGURE 4C). Based on this analysis, a NT_{50} of 1:32 equals 944 EU (dashed line, FIGURE 4A) and indicates that ~50% of volunteers at >20 years after a single vaccination have neutralizing antibody titers of ≥1:32. Neutralizing antibodies were below detection (NT_{50}<1:4) in 16/16 samples from unvaccinated volunteers (data not shown).

An important point to consider is whether or not high antibody responses are correlated with increased levels of T cell memory, since this would shed new light on whether high neutralizing antibody titers were directly involved with protective immunity against smallpox or whether they are simply a surrogate marker indicative of increased antiviral T cell responsiveness. Therefore, antiviral T cell responses were compared to their accompanying antibody levels in individuals who had been vaccinated ≤7 years previously (*i.e.*, a cohort similar to Mack et al., *(supra)*[8]) (FIGURE 4D) as well as in individuals vaccinated 14–40 years ago, or 41–75 years ago (*i.e.*, cohorts similar to contemporary populations) (FIGURES 4E and 4F).

Significantly, no correlation was observed between virus-specific T cell levels and antibody titers at early or late time points, thus indicating that humoral and cellular immunity are independently regulated. Because the early cohort (≤7 years post-vaccination) is reasonably comparable to the smallpox contacts examined by Mack et al., (*supra*)[8], the results indicate that high neutralizing antibody titers are still an effective biomarker of protective immunity, but not necessarily indicative of enhanced T cell memory.

Significantly, this also indicates that high neutralizing antibody levels have a more direct role in protective immunity against smallpox than previously realized.

EXAMPLE IV

(Application of the inventive SABRE platform; monkeypox virus-specific immune response was detected)

The inventive SABRE (systematic analysis of biologically relevant epitopes) platform, as described herein above and as exemplified in this EXAMPLE, enables rapid and effective mapping/identification of biologically relevant (*e.g.*, immunodominant) polypeptide epitopes suitable for diagnostic and/or therapeutic applications.

Using SABRE, a representative serological assay was developed that can be used to determine whether or not a person has been infected with the monkeypox virus, a dangerous orthopoxvirus on the U.S. government's Select Agent list, and a possible pathogen that might be used for bioterrorism. Additionally, a dual assay for determining both virus infection, and virus-specific immune response is provided. Furthermore, vaccines agents are provided, based on the SABRE-identified antigens (proteins/polypeptides/epitopes).

Specifically, using published sequences of several strains of vaccinia, monkeypox (MPV), cowpox, and Variola (smallpox), monkeypox-specific genes were identified that are not encoded in the genome of vaccinia—the most common poxvirus that, for example, Americans are likely to have pre-existing immunity against. Overlapping peptide reagents were ordered and obtained (from Mimotopes) that spanned the entire protein of several of these genes (*e.g.*, MPV genes: D2L, B18R, N2R and N3R), as well as to several likely candidates in the MPV B21R gene.

ELISA plates were coated with individual peptides and then tested the reactivity of serum samples from subjects with verified MPV infections, possible sub-clinical MPV infections, and negative controls including subjects recently immunized with vaccinia or subjects that have no known exposure to orthopoxvirus infections.

Based on this analysis, several unique peptide epitopes were identified that are immunogenic and recognized by MPV convalescent serum (5/5 = positive), but not by negative control serum samples from subjects that have not been infected with MPV (0/4 = positive). The preferred immunogenic polypeptides are summarized in TABLE 2 below.

Preferably, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2R), 10 (MPV N3R), 16 (MPV B18R) and 20 (MPV B21R), and epitope-bearing fragments of SEQ ID NOS:1 (MPV D2L), 6 (MPV N2R), 10 (MPV N3R), 16 (MPV B18R) and 20 (MPV B21R).

More preferably, the monkeypox polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:2-5 (MPV D2L), 7-9 (MPV

N2R), 11-15 (MPV N3R), 17-19 (MPV B18R) and 21-29 (MPV B21R), and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19 and 21-29.

More preferably, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:10 (MPV N3R) and 20 (MPV B21R), and epitope-bearing fragments of SEQ ID NOS:10 and 20.

More preferably, the monkeypox protein or polypeptide comprises at least one epitope of a sequence selected from the group consisting of SEQ ID NOS:11-15 (MPV N3R) and 21-29 (MPV B21R), and epitope bearing fragments of SEQ ID NOS: 11-15 and 21-29.

More preferably, the epitope comprises a sequence selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$) and 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

According to aspects of the present invention, these peptides are used in diagnostic kits and assays to detect virus immune response (*e.g.*, virus-specific serum antibodies).

Dual determination of infection and monkeypox virus-specfic immunity

As described above, aspects of the present invention provide methods to identify monkeypox-specific immune response by screening the serum of subject for monkeypox-specific antibody. The window for such screening is very broad, because people continue to make antibodies to orthopoxviruses for decades. Nonetheless, such assays may not be sensitive enough to identify infected subjects at very early time points of infection, especially if an immune response has not yet been mounted.

According to the present invention, a Dual Detection System (DDS) overcomes this limitation. Preferably, the DDS is used to simultaneously (in parallel) identify either the orthopoxvirus (*e.g.*, monkeypox), or the immune response against the virus (*e.g.*, monkeypox-specific antibody).

For example, monkeypox-specific antibody responses, as describe above, are identified by initially screening a library of monkeypox peptides to identify peptides only recognized by serum samples from monkeypox infected patients. Monoclonal antibodies (*e.g.*, mouse) are then developed against the unique peptide sequences identified in the

infected serum screen. These antibodies have utility to detect the monkeypox virus, based on the fact that these peptides are very specific and non-cross-reactive.

Kits. According to preferred aspects of the invention, screened biologically relevant peptides are used, for example, as part of a 'dip-stick' kit to detect orthopoxvirus-specific serum antibodies by about 6 to 10 days after infection, and as late as 75-years after infection.

Additionally, because the highest virus titers are likely to precede the strongest antibody responses, the respective (cognate) monkeypox-specific monoclonal antibody are used, for example, as part of a 'dip-stick' kit to detect orthopoxvirus infection.

Therefore, the inventive dual assay approach broadens the window of detection to include the first signs of clinical symptoms. The DDS approach allows the broadest, yet highly specific identification of the pathogen of interest. By detecting the virus directly, positive results are obtained at early time points, before antibody responses have had time to be mounted. By detecting the virus-specific antibodies in parallel, positive results are obtained even in people that are recovering (or have recovered) from the infection (*i.e.*, where there is no virus to find) or had such low virus titers to begin with that they scored negative by the direct virus-detection approach.

TABLE 2. Summary of exemplary monkeypox virus proteins, along with exemplary preferred polypeptides thereof.

Preferred Monkepox Virus Protein	Accession Number	Preferred Polypeptide	Polypeptide Sequence	SEQ ID NO:
D2L	AAL40463			SEQ ID NO:1
D2L		1-20	MYYANICLDFDNNVYTVKDK	SEQ ID NO:2
D2L		11-30	DNNVYTVKDKNYTNAVIEYP	SEQ ID NO:3
D2L		21-40	NYTNAVIEYPVVCNFRRYSE	SEQ ID NO:4
D2L		31-50	VVCNFRRYSESDSDVDDRAE	SEQ ID NO:5
N2R	NP_536613			SEQ ID NO:6
N2R		1-20	MQYLNETDNLGNTVLHTHIF	SEQ ID NO:7
N2R		21-40	LDYISLKICKRYISHKYPLC	SEQ ID NO:8
N2R		31-50	RYISHKYPLCNIINGYIDNT	SEQ ID NO:9
N3R	NP_536614			SEQ ID NO:10
N3R		21-40	CHKLVHYFNLKINGSDITNT	SEQ ID NO:11
N3R		91-110	SSQYEELEYYYSCDYTNNRP	SEQ ID NO:12
N3R		111-130	TIKQHYFYNGEEYTEIDRSK	SEQ ID NO:13
N3R		151-170	DSEDCIIYLRSLVRRMEDSN	SEQ ID NO:14
N3R		157-176	IYLRSLVRRMEDSNKNSKKT	SEQ ID NO:15

Preferred Monkepox Virus Protein	Accession Number	Preferred Polypeptide	Polypeptide Sequence	SEQ ID NO:
B18R	NP_536606			SEQ ID NO:16
B18R		31-50	YFKTMFTTPMIARDLATRVN	SEQ ID NO:17
B18R		41-60	IARDLATRVNLQMFDKMPSK	SEQ ID NO:18
B18R		51-70	LQMFDKMPSKILYSTYTIGI	SEQ ID NO:19
B21R	NP_536609			SEQ ID NO:20
B21R		150-169	PLPTSAVPYDQRSNNNVSTI	SEQ ID NO:21
B21R		195-214	DTVDNNTMVDDETSDNNTLH	SEQ ID NO:22
B21R		362-381	IRNSVSTTNSRKRRDLNGEF	SEQ ID NO:23
B21R		669-688	TRKGATRRRPRRPTNDGLQS	SEQ ID NO:24
B21R		684-703	DGLQSPNPPLRNPLPQHDDY	SEQ ID NO:25
B21R		699-718	QHDDYSPPQVHRPPTLPPKP	SEQ ID NO:26
B21R		729-748	PVGQLPPPIDQPDKGFSKFV	SEQ ID NO:27
B21R		805-824	KNNVPVIGNKHSKKYTSTMS	SEQ ID NO:28
B21R		842-861	TRSTTLSRKDQMSKEEKIFE	SEQ ID NO:29

EXAMPLE V

(Inventive assays were used to show that cross-protective antiviral immunity against West African monkeypox can be maintained for decades after smallpox vaccination)

This Example shows, according to particular aspects, independent and internally validated diagnostic approaches with ≥95% sensitivity and ≥90% specificity for detecting clinical monkeypox infection. Applicants detected, *inter alia*, three previously unreported cases of monkeypox in pre-immune individuals at 13, 29, and 48 years post-smallpox vaccination who were unaware that they had been infected because they were spared any recognizable disease symptoms. Together, this shows that the U.S. monkeypox outbreak was larger than previously realized and more importantly, indicates that cross-protective antiviral immunity against West African monkeypox can be maintained for decades after smallpox vaccination.

Rationale. Approximately 50% of the U.S. population has received smallpox vaccinations before routine immunization ceased in 1972 (civilians) or 1990 (military personnel). There is a question as to whether any potential residual immunity would translate into full protection against the onset of orthopoxvirus-induced disease. The U.S. monkeypox outbreak of 2003 provided the opportunity to examine this critical issue.

Following hospitalization of the first monkeypox victim, ~2 weeks elapsed before the outbreak was identified by local health officials and the CDC[9]. The current diagnostic algorithm for detection of smallpox[10] was apparently not utilized since there were several instances of febrile patients with smallpox-like lesions who were misdiagnosed and discharged from the hospital (11). Similar failures in diagnosis occurred during a recent smallpox outbreak drill (12). If the monkeypox outbreak had actually been smallpox, the outcome might have been very different, with secondary infections and further spread likely to have occurred during the prolonged period needed to identify the outbreak. Current diagnostic tests, which aim to diagnose an acute infection, rely on the presence of virus (including virus-specific PCR; polymerase chain reaction) and thus approximately half of the 71 reported monkeypox cases remain unconfirmed (9) because the results have scored negative or equivocal. Moreover, there is a critical need for better diagnostics for investigating monkeypox outbreaks in Africa (13). One objective of this study was to develop novel and improved diagnostic methods for identifying rare orthopoxvirus infections.

Applicants have demonstrated herein that antiviral antibody and T cell responses could be maintained for up to 75 years after smallpox vaccination (see also 14). Since ~50% of the U.S. has been vaccinated against smallpox, it is critical to determine whether or not the antiviral immunity identified in these studies might translate into protective immunity. The 2003 monkeypox outbreak provided a natural virus-challenge experiment that offers insight into this important question. The results presented herein identify three previously unreported cases of clinically inapparent monkeypox infections in which individuals who received the smallpox vaccine 13–48 years previously still maintained fully protective cross-reactive immunity and were spared any observable disease symptoms after exposure to an infectious dose of a West African strain of monkeypox. According to particular aspects, these individuals maintained protective cross-reactive immunity.

Methods:

Subjects. Recruitment of adult volunteers (n=44) was conducted in the state of Wisconsin since this was the epicenter of the U.S. outbreak and represented the largest

concentration of monkeypox individuals within a small geographical region (9). Subjects were screened and only those with close contact with monkeypox patients or infected prairie dogs were included in the study. Subjects who claimed to have had overt monkeypox disease symptoms provided us with authorization to confirm this with their primary physician. Subjects were categorized as suspect, probable, or confirmed cases of monkeypox based on the diagnostic and epidemiological criteria set forth by the CDC (28). Other control subjects consisted of Oregon residents (n=21) who had been recently vaccinated against smallpox or who had provided multiple serum samples at 33–37 years post-vaccination. Each subject provided informed written consent before signing HIPAA-compliant research authorization forms, filling out a medical history questionnaire, and providing a 50–100 mL blood sample that was processed at OHSU. Peripheral blood mononuclear cells (PBMC) were cryopreserved in aliquots and stored in liquid nitrogen. Plasma and serum samples were stored at –20°C or –80°C. All clinical studies were approved by the Institutional Review Board of Oregon Health & Science University.

Intracellular cytokine staining (ICCS). Intracellular cytokine staining was performed as previously described (14). Briefly, PBMC were cultured at 37°C with 6% CO_2 in RPMI containing 20 mM HEPES, L-glutamine, antibiotics, and 5% heat-inactivated FBS (Hyclone), with or without vaccinia virus (sucrose gradient-purified intracellular mature virus (IMV), vaccinia strain Western Reserve) at an MOI of 0.1. After 12 hours of culture, Brefeldin A (ICN) was added at a final concentration of 2 μg/mL for an additional 6 hours. The cells were stained overnight at 4°C with antibodies specific for CD8β (clone 2ST8.5H7, Beckman Coulter) and CD4 (clone L200, PharMingen). Cells were fixed, permeabilized and stained intracellularly using antibodies to IFNγ (clone 4S.B3) and TNFα (clone Mab11), both from PharMingen. Samples were acquired on an LSRII instrument (Beckton Dickinson) using FACSDiva software (Beckton Dickinson), acquiring 1–2 million events per sample. Data was analyzed using FlowJo™ software and a live cell gate was performed using forward and side scatter characteristics. The number of $IFN\gamma^+TNF\alpha^+$ T cells was quantitated after first gating on live $CD4^+CD8^-$ or $CD4^-CD8^+$ cells and subtracting the number of $IFN\gamma^+TNF\alpha^+$ events from uninfected cultures. Each assay contained PBMC from a positive control (~1

year post-smallpox vaccination), which scored 775±188 $IFN\gamma^+TNF\alpha^+CD4^+$T cells per 10^6 $CD4^+$ T cells and 1,844±585 $IFN\gamma^+TNF\alpha^+CD8^+$T cells per 10^6 $CD8^+$ T cells, respectively. One or more negative controls consisting of PBMC from vaccinia-naïve subjects were included in each assay.

ELISA. Vaccinia-specific and monkeypox-specific ELISA assays were performed as described herein (see also 14) using vaccinia (strain: WR) or monkeypox (strain: Zaire) whole virus lysate (inactivated by pretreatment with 3% H_2O_2 for >2 hours). An internal positive control was included on each plate to normalize ELISA values between plates and between assays performed on different days. Antibody titers were determined by log-log transformation of the linear portion of the curve, with 0.1 optical density (O.D.) units used as the endpoint and conversion performed on final values. Note: the same positive control sample (~1 year post-smallpox vaccination) was used on both vaccinia and monkeypox-coated plates and normalized to the same ELISA value (e.g. normalized to 10,000 EU for each type of ELISA).

Peptide-specific ELISA assays were performed by coating 96-well flat-bottomed plates with a different 20-mer peptide (2 μg/mL in PBS) in each well. A number of candidate peptides were identified based on the monkeypox genome (accessed via the Poxvirus Bioinformatics Resource Center: http://www.poxvirus.org/). Peptides were purchased from Mimotope as 20mers with 10 amino acid overlap. Each peptide (~2 mg) was dissolved in 200 μL DMSO (Sigma, ACS spectrophotometric grade) followed by the addition of 200 μL of water (HPLC grade) for a final master stock concentration of ~5 mg/mL. Inactivated vaccinia lysate was added to one well (functioning as a positive control for vaccinia-immune or monkeypox-immune samples and as a negative control for orthopoxvirus-naïve samples) and human plasma (containing IgG) was used to coat one well on each plate as an additional positive control. A single dilution (1:50) of plasma or serum was added to preblocked plates and incubated for 1 hour. After washing, plates were incubated for 1 hour with horseradish peroxidase-conjugated polyclonal goat antibodies to human IgG(γ) (Jackson ImmunoResearch Laboratories, Inc.). After an additional washing step, detection reagents were added, followed by 1M HCL, and the plates were read on an ELISA plate reader.

Samples were considered positive for a particular peptide if they scored ≥2-fold over background on at least 2 to 3 different ELISA plates.

RESULTS:

A common misconception of the U.S. monkeypox outbreak is that infection required direct contact or direct inoculation through scratches/bites in order for monkeypox to be transmitted from infected prairie dogs to humans. Upon interviewing monkeypox patients in Wisconsin, applicants uncovered several cases in which monkeypox was transmitted to humans by indirect contact, possibly in the form of fomites or aerosol exposure (TABLE 3).

With respect to TABLE 3, subjects were asked to fill out a medical history questionnaire in which they described their smallpox vaccination status, disease symptoms, and location in which their exposure to monkeypox likely occurred. The 12 subjects in the upper portion of the table had not received smallpox vaccination whereas the 8 subjects in the lower portion of the table had received smallpox vaccination (typically confirmed by identification of smallpox vaccination scar on the left arm) and the estimated number of years after smallpox vaccination is listed (NA, not applicable). Questions regarding the symptoms of monkeypox infection were based on clinical diagnostic criteria set forth by the CDC during the outbreak[28] and consisted of rash (macular, papular, vesicular, or pustular; generalized or localized; discrete or confluent), fever (subjective or measured temperature ≥37.4°C) or other common symptoms including; headache, backache, swollen lymph nodes (lymphadenopathy), sore throat, cough, and shortness of breath. Subject #453 reported no rash, but exhibited all other monkeypox disease symptoms, some of which were moderate to severe. Subject #500 reported only one monkeypox lesion at the puncture site after being bitten by an infected prairie dog. Subjects #446, #449, and #455 reported no symptoms. Each subject reported exposure to monkeypox-infected prairie dogs in their homes or their place of work (see FIGURE 8 and references (9, 11, 28-30) for further description of the locations of monkeypox exposure). Location of exposure abbreviations: PS2; Pet store 2, Dist; prairie dog distributor, VC2; Veterinary Clinic 2, SEHH; Southeastern household, NWHH; Northwestern household, PS1; Pet store 1, VC3; Veterinary Clinic 3 is not shown in

FIGURE 8 but is located in NW Wisconsin and was a location in which an ill prairie dog from the NWHH was treated. Putative route of exposure abbreviations: Direct C; Exposure by direct contact or handling of an infected prairie dog, Indirect C/A: Exposure by indirect contact with infected prairie dog or by possible aerosol exposure. In these cases, the subjects entered rooms in which infected prairie dogs were present, or had been present, but did not have direct contact with the infected animals.

In one case, a subject contracted monkeypox after an infected prairie dog was carried into her home when she was not present. The animal was apparently not placed on the floor or furniture and yet this subject, who had no other contact with monkeypox patients or prairie dogs, still contracted the disease. Cases of monkeypox in subjects who had not directly handled infected prairie dogs also occurred at other sites including a veterinary clinic in which a number of subjects contracted the disease by being present in (or later entering) a room in which an infected prairie dog was nebulized. Although applicants found no evidence of direct human-to-human spread in our limited assessment of this monkeypox outbreak, these results imply a word of caution to clinicians and other health care workers who might encounter virulent orthopoxviruses.

TABLE 3 shows the reported symptoms, vaccination status, and putative route of exposure for 12 cases of monkeypox in unvaccinated individuals, 5 cases of monkeypox in subjects who had previously received smallpox vaccination, and 3 cases of clinically inapparent monkeypox in previously vaccinated individuals. A comparison of the number of monkeypox lesions reported by vaccinated and unvaccinated subjects can be found in FIGURE 9.

FIGURE 9 shows a comparison of the number of monkeypox lesions reported by unvaccinated and vaccinated monkeypox patients. Subjects were asked to fill out a medical history questionnaire describing their history of monkeypox infection including the number of monkeypox lesions or "pocks" that developed during the course of this acute viral infection.

Based on retrospective self-reporting, it was unclear if the overall extent of other disease symptoms were modified in subjects who had been previously vaccinated. In our view, quantitation of the number of monkeypox lesions represented the least subjective symptom described in the medical history questionnaire. Based on the results from all monkeypox-infected subjects, there was an average of 33 monkeypox lesions in the unvaccinated group versus an average of 3.6 lesions in the vaccinated group, representing nearly a 10-fold difference overall. If we exclude the most severe case of monkeypox from the unvaccinated cohort (a patient with >200 monkeypox lesions and severe symptoms requiring hospitalization) and exclude the three clinically asymptomatic cases of monkeypox from the previously vaccinated cohort, then there were still significantly more monkeypox lesions observed in the unvaccinated subjects than in the vaccinated cohort (average = 18 vs. 5.6 monkeypox lesions, respectively)($P = 0.045$, ANOVA).

This result is suggestive, but not necessarily correlative, of partial protection due to pre-existing immunity and is consistent with anecdotal evidence from another report indicating that vaccinated individuals may have a milder course of disease following monkeypox infection due to pre-existing immunity from childhood smallpox vaccination. In this case report of one family (Sejvar, J. J. et al. Human monkeypox infection: a family cluster in the midwestern United States *J Infect Dis* **190**, 1833-40 (2004)), an unvaccinated mother exhibited ~200 monkeypox lesions and her unvaccinated 6-year-old daughter exhibited ~90 monkeypox lesions (in addition to severe encephalitis resulting in a coma that lasted for 12 days), whereas the previously vaccinated father developed just 2 monkeypox lesions and experienced only mild, flu-like symptoms for ~48 hours.

In addition to the subjects in TABLE 3, blood samples were also obtained from 24 other subclinical contacts in Wisconsin (referred to as naïve contacts and vaccinia-immune contacts) as well as from vaccinia-naive and vaccinia-immune subjects who reside in Oregon.

Current tests used to help diagnose/confirm monkeypox infection are based solely on virus identification; this is a method of limited utility after the acute infection has cleared. Thus, as seen in the U.S. monkeypox outbreak in 2003 wherein about half of the reported

cases of monkeypox remain unconfirmed (9), there is a need for reliable tests that can retrospectively define the scope of an outbreak.

Applicants took an immunological approach to monkeypox diagnostics by initially performing ELISA assays using inactivated whole-virus lysates as a first attempt at discriminating monkeypox patients from uninfected contacts (FIGURES 5A-5D)).

Figures 5A-5D show antiviral antibody responses following orthopoxvirus infection. (A) Serum samples were drawn between 2 months to 1 year post-infection/exposure and tested on ELISA plates coated with equivalent amounts of inactivated vaccinia or monkeypox viral antigen. The monkeypox:vaccinia antibody ratio was determined by dividing the monkeypox-specific titers by the vaccinia-specific antibody titers (e.g. 2,000 EU against vaccinia and 4,000 EU against monkeypox results in a ratio of 2.0). A ratio could not be accurately determined on samples that scored <30 EU against vaccinia since these scores were below the limit of detection by this assay. (B) Antiviral antibody titers declined rapidly (mean: 62% decline, range: 35–85%) after recent monkeypox infection, even in the three subjects with clinically inapparent infections (dashed lines). (C) Similar results were observed in control subjects examined at similar defined time points following recent smallpox revaccination involving a live vaccinia virus infection (mean: 57% decline, range 7-76%). (D) In contrast, long-term antiviral antibody responses remained largely unaltered (mean: <0.5% annual decline, range: 0-3%), indicating that rapid antibody decline only occurs during the early stages after a recent orthopoxvirus infection. Each ELISA was repeated 2–6 times and symbols represent mean titers with error bars representing standard deviation. The numbers, 446, 449, and 455, represent the subject ID numbers of individuals with clinically inapparent monkeypox infections.

TABLE 3. Summary of prior vaccination status, symptoms, and exposure to monkeypox.

ID#	Years after smallpox vaccination	Rash	Fever	Headache	Backache	Swollen lymph nodes	Sore throat	Cough	Shortness of breath	Location of exposure	Putative route of exposure
447	NA	X	X	X	X		X	X	X	PS2	Bite
452	NA	X	X	X		X	X	X		Dist	Bite/Scratch
453	NA		X	X	X	X	X	X	X	Dist	Direct C
461	NA	X	X	X		X	X	X	X	VC2	Indirect C/A
462	NA	X	X	X	X	X	X	X	X	VC2	Direct C
473	NA	X	X	X			X	X	X	VC2	Scratch
481	NA	X	X	X	X	X	X		X	VC2	Direct C
482	NA	X	X	X	X	X	X			VC2	Indirect C/A
484	NA	X	X	X		X	X	X	X	VC2	Direct C
489	NA	X	X	X		X	X			SEHH	Indirect C/A
519	NA	X	X			X	X			NWHH	Existing scratch
520	NA	X	X			X	X			NWHH	Direct C
450	34	X		X	X	X	X	X		SEHH	Scratch
451	32	X	X	X	X	X	X	X	X	SEHH	Direct C
454	38	X	X	X	X	X	X	X	X	PS1	Direct C
463	38	X		X		X				VC1	Existing cut
500	34	X	X	X		X				VC3	Bite
446	38									PS2	Indirect C/A
449	13									SEHH	Direct C
455	29									PS1	Direct C

Vaccinia ELISA assays exhibit 98% sensitivity and 100% specificity for detecting antiviral immunity, with antibody titers of unvaccinated individuals residing below 100 ELISA units (EU) (14). Diagnosing monkeypox in subjects under the age of 35 (*i.e.* born after routine smallpox vaccination was abandoned) was straightforward because unvaccinated contacts exhibited negligible antibody titers (<100 EU against vaccinia or monkeypox, n = 12). In contrast, 12/12 of monkeypox patients demonstrated antibody titers ranging from 1,279–9,765 EU against vaccinia and 5,815–21,147 EU against monkeypox. Notably, these subjects exhibited high antibody titers that typically scored 2- to 4-fold higher against monkeypox than vaccinia. Although there is substantial cross-reactivity between orthopoxviruses, this indicates that additional antibody epitopes exist in monkeypox. This is consistent with earlier studies in which monkeypox-specific antibodies were still detected after cross-adsorption to vaccinia antigens (15-17).

Serological analysis of individuals over age 35 is more challenging to interpret because >90% of Americans over this age have been immunized and maintain lifelong vaccinia-specific antibody responses (18). Vaccinated contacts had antibody titers ranging between 123–4,408 EU against vaccinia and approximately a 1:1 ratio when comparing antibody titers against vaccinia versus monkeypox (FIGURE 5A). In contrast, monkeypox infection of vaccinated subjects resulted in more heterogeneity. These subjects typically demonstrated high antibody titers against vaccinia and/or strong antibody titers to monkeypox, resulting in a high monkeypox:vaccinia ratio. In particular, three previously vaccinated subjects who had experienced no clinical symptoms of monkeypox (#446, #449, and #455 at 43, 13, and 29 years post-vaccination, respectively), demonstrated exceptionally high serological responses that were indicative of recent orthopoxvirus infection and could be clearly distinguished from uninfected vaccinia-immune contacts. This differential monkeypox:vaccinia ELISA approach provided the first indication that applicants had identified subjects with fully protective immunity against monkeypox.

If these serological results are indicative of recent monkeypox infection, then antibody titers should drop sharply after viral clearance. For this analysis, applicants compared antiviral antibody responses at early (2–4 months) and late (1 year) time points after monkeypox

infection and found that antibody titers declined by ~60%. This is strikingly similar to antibody decline following booster smallpox vaccination which is included here as a positive control (FIGURE 5C). In contrast, long-term immunity following childhood vaccination is stable and averaged <1% decline over a 1–2 year period (FIGURE 5D). Using >100 EU and >30% decline in antibody between paired acute and convalescent serum as diagnostic criteria indicative of recent orthopoxvirus infection, applicants achieved 100% (20/20) sensitivity for monkeypox detection, 92% (12/13) sensitivity for recent vaccinia infection/vaccination, and 100% specificity (0/8 long-term vaccinia-immune serum samples declined by 30% and 0/12 naïve contacts exhibit virus-specific antibodies >100 EU, FIGURE 5A). These results provided compelling evidence that the three individuals with asymptomatic infections were indeed infected with monkeypox since they had antibody titers that declined rapidly, an expected result following recovery from a recent orthopoxvirus infection.

Intracellular cytokine staining analysis (ICCS) was used in monkeypox diagnosis by quantitating orthopoxvirus-specific $CD4^+$ and $CD8^+$ T cell responses (FIGURE 6). Using a diagnostic cut-off of 200 $IFN\gamma^+TNF\alpha^+CD8^+$ T cells/10^6 $CD8^+$ T cells, applicants achieved 95% sensitivity (19/20 monkeypox-infected subjects scored >200) and 100% specificity (0/12 naïve contacts and 0/12 vaccinia-immune contacts scored >200).

Specifically, FIGURE 6 shows diagnosis of recent monkeypox infection by quantitation of orthopoxvirus-specific T cells. The frequency of virus-specific T cells capable of producing both IFNγ and TNFα after direct *ex vivo* stimulation with vaccinia virus was determined by intracellular cytokine staining (ICCS). Samples that scored below detection were graphed with values of <1 per 10^6. The vertical dashed line represents the diagnostic cut-off of virus-specific $CD8^+$ T cells used for distinguishing recently infected monkeypox patients from uninfected naïve contacts and vaccinia-immune contacts (immunized >20 years previously). This data and comparison with previous studies with a large number of vaccinia-immune subjects[14], indicates that this approach provides ≥95% sensitivity and >97% specificity for detecting a recent orthopoxvirus infection.

In previous work (14), <4% (0/26) of vaccinia-naïve subjects and <3% (7/256) of vaccinia-immune subjects at >20 years post-vaccination exhibited antiviral responses of ≥200

$IFN\gamma^+TNF\alpha^+CD8^+$ T cells/10^6 $CD8^+$ T cells – indicating this diagnostic cut-off has 100% specificity in vaccinia-naïve subjects and >97% specificity overall. Two of three asymptomatic individuals (#446 and #455) showed high T cell levels comparable to subjects with clinical symptoms, again verifying their recent monkeypox infection. Only one monkeypox-infected subject (#449) did not clearly segregate with other monkeypox patients, but this subject's virus-specific T cell responses were still proportionally high and, if similar to acute vaccinia infection (19), may have been higher if a blood sample could have been obtained earlier than 3 months post-exposure. This is the first demonstration of a T cell-based diagnostic approach capable of distinguishing 100% of clinically-apparent monkeypox-infected individuals from naïve and vaccinia-immune contacts and provides independent confirmation of applicants' initial serological results.

Many monkeypox-infected patients exhibited higher antibody responses against monkeypox than against vaccinia, suggesting the existence of novel epitopes (FIGURE 5). To identify potential epitopes, candidate genes were identified in monkeypox (20) that are not present in the vaccinia genome, including D2L, B18R, N2R, N3R, and B21R and overlapping peptides were used for screening linear antibody epitopes (FIGURE 7A-7C).

Specifically FIGURE 7 shows analysis of monkeypox-specific peptide ELISA assays for diagnosing monkeypox infection. The numbers on the X axis are the exemplary peptide numbers (peptide #1 is amino acids 1-20, peptide #2 represents amino acids 10-30, peptide #3 represents amino acids 20-40, etc.) and each peptide is 20 amino acids long and overlaps the previous peptide by 10 amino acids. Exemplary peptide #67, for example, represents B21R amino acids 660-680. Serum or plasma samples (1:50 dilution) obtained at 2 months to 1 year post-infection/exposure were incubated on ELISA plates coated with an individual peptide in each well. Samples were scored positive for a particular peptide if they scored ≥2-fold over background on at least 2 to 3 different ELISA plates. Panels A-C show the percentage of samples that scored positive against peptides from putative monkeypox proteins, D2L, B18R, N2R, N3R, and B21R. A, Primary monkeypox (n = 12), B, Vaccinia – monkeypox (n = 8), C, Monkeypox contacts, vaccinia-naive and vaccinia-immune (n = 20 to 24). The major immunodominant peptide epitopes are marked with one asterisk (*) for those with ≥90%

specificity or with two asterisks for peptide epitopes with 100% specificity. Monkeypox contacts were evenly divided between vaccinia-naïve and vaccinia-immune subjects.

Of the smaller putative gene products (D2L, B18R, N2R, and N3R), the carboxy-terminus of the N3R gene was modestly promising with 67% sensitivity for identifying unvaccinated monkeypox cases, 38% sensitivity for vaccinated monkeypox cases, and 88% specificity among uninfected naïve or vaccinia-immune contacts. The larger B21R gene product (1,879 amino acids) was highly immunogenic, with 100% of monkeypox-infected subjects responding to ≥3 epitopes and 60% of these subjects responding to ≥10 peptides (range: 3–41 peptides). The most immunogenic B21R epitope was peptide #185, which elicited 100% (12/12) sensitivity in unvaccinated monkeypox patients, 50% (4/8) sensitivity in vaccinated monkeypox patients, and 90% specificity (Figure 3). Of the asymptomatic individuals unknowingly infected with monkeypox, Subject #446 had the highest overall antibody response (FIGURE 5A) and the strongest $CD4^+$ T cell response (Figure 2), but responded to only four B21R epitopes. However, this individual responded to B21R peptides #126 and #180, both of which exhibit reasonably high specificity (80% and 85%, respectively). Subject #455 responded to five B21R epitopes including peptide #20 and #148 (100% and 95% specificity, respectively) and Subject #449 responded to nine B21R epitopes including peptide #20 and #115 (100% and 95% specificity, respectively). Together, these results indicate that linear peptides provide an effective and sensitive approach to monkeypox diagnostics.

There were 39 reported cases of monkeypox in Wisconsin; 18 laboratory-confirmed, and 11 described by Reed *et al.* (11). Of these subjects, we found 100% concordance with the diagnostic results obtained from monkeypox patients with clear clinical disease symptoms and who were positive by virological assays such as electron microscopy (EM), viral culture (VC), immunohistochemistry (IHC) and/or PCR (n=7). In addition, applicants' approach confirmed three probable/suspect cases of monkeypox described in this earlier study (11) who had exposure to monkeypox, demonstrated most or all clinical symptoms of monkeypox, but who were negative by virological analysis (FIGURE 8). The relationship between the three previously unidentified individuals who experienced asymptomatic monkeypox infections can now be placed into the context of the greater monkeypox outbreak.

FIGURE 8 shows the relationship between reported and unreported (i.e. asymptomatic) monkeypox infections. This figure was modified from a similar flow-chart diagram published by Reed *et al.* (11) and shows the relationship between different monkeypox survivors in the context of the WI monkeypox outbreak. Patients 4 and 5 are subjects who purchased 39 prairie dogs from an Illinois distributor and sold 2 prairie dogs to the family in the Northwestern WI household, the site of the first recorded case of human monkeypox in the United States. Two prairie dogs were sold to Pet store 1, 10 prairie dogs were sold to Pet store 2, and an ill prairie dog was treated at Veterinary Clinic 1. Pet store 2 then sold a prairie dog to subjects in a Southeastern WI household and when the animal showed disease symptoms, it was treated at Veterinary Clinic 2. Further details of the outbreak are published elsewhere[9,11,28-30]. The diagnostic methodology used by Reed *et al.* and the new immunological techniques developed here are provided for comparison. The previous diagnostic criteria involved virological techniques including electron microscopy (EM), viral culture (VC), immunohistochemistry (IHC), and polymerase-chain-reaction (PCR). Our study used diagnostic procedures including vaccinia whole-virus ELISA with a positive titer (i.e. >100 EU) followed by >30% decline in antibody titers between paired acute and convalescent serum as diagnostic criteria indicative of a recent orthopoxvirus infection (Orthopox-ELISA). Intracellular cytokine staining (ICCS) was used to quantitate orthopoxvirus-specific T cells, with >200 antiviral $CD8^+$ T cells/10^6 as diagnostic criteria indicative of monkeypox infection. Monkeypox B21R peptide ELISA (Pep-ELISA) results were considered positive for monkeypox infection if responses were observed against one or more B21R peptides that have ≥90% specificity and ≥90% sensitivity. Samples were labeled as Unconfirmed if they were previously listed as probable or suspect cases according to CDC criteria[28]. In these cases, the virological diagnostic methods scored negative or equivocal even though the subjects had known contact with monkeypox-infected prairie dogs and experienced many or all of the characteristic disease symptoms of monkeypox infection. The previously reported cases of clinically apparent monkeypox are shown in rectangles and the three asymptomatic monkeypox cases identified in this current study are shown in ovals.

In summary of this EXAMPLE V, applicants used a multi-faceted approach to diagnosing clinically apparent and inapparent monkeypox infections. Using an optimized differential vaccinia:monkeypox whole-virus ELISA test, applicants identified subjects with recent monkeypox infection and these results were confirmed by quantitation of antibody decay rates during the first year following recovery. A cell-mediated diagnostic approach was also developed wherein applicants measured the number of orthopoxvirus-specific T cells by ICCS. This proved to be an effective, independent approach capable of correctly diagnosing all clinically apparent monkeypox patients and 2/3 inapparent monkeypox infections. Serological studies using overlapping peptides revealed the monkeypox B21R protein as an important antibody target with several key immunodominant epitopes. These peptide reagents worked well in retrospective serological analysis of a monkeypox outbreak and have utility for the development of monkeypox-specific monoclonal antibodies suitable for rapid, direct detection of the virus. Similar technology could potentially be developed to detect and/or monitor a deliberate smallpox attack.

Applicants'diagnostic approaches confirmed monkeypox infection in patients who were previously listed as probable or suspect (FIGURE 8). These patients demonstrated multiple disease symptoms indicative of monkeypox, but tested negative or equivocal by current virological techniques. In some cases, this may have been due to subjects not immediately seeking medical attention, and after the infection had resolved, virological assays such as PCR would no longer be capable of making a positive diagnosis. Although orthopoxvirus-specific PCR can detect as few as 25 genome equivalents in the laboratory (21), it only detected monkeypox in 6/11 cases (55% sensitivity) of clinically overt monkeypox (11). An advantage of using the immunological assays described here is that a positive diagnosis can be made retrospectively due to persisting immunity. Monkeypox continues to be a problem in Africa, with outbreaks that are difficult to monitor due to inconsistencies in epidemiological methodology and the limitations of current diagnostics (13). Antiviral antibody and T cell responses begin to rise at or near the time of disease onset, so novel and highly sensitive immunological techniques may potentially prove effective for monkeypox diagnosis during an

ongoing outbreak, but further studies are necessary to determine the earliest time in which monkeypox infection can be reliably detected by these methods.

If this study was performed in Europe where cowpox is endemic (22-24), or in Africa where monkeypox outbreaks occur (2-6), then one could not rule out the possibility that long-term immunity was due to intermittent re-exposure to crossreactive orthopoxviruses. This is unlikely to be an issue in the instant study because the last case of smallpox in the U.S. was in 1949, routine smallpox vaccination was discontinued in 1972, and there are no orthopoxviruses indigenous to North America that are known to infect humans. Thus, the U.S. monkeypox outbreak provided a rare opportunity to measure protective antiviral immunity in the absence of endemic orthopoxviruses.

Greater than 100 million Americans have received smallpox vaccination and a question arising from this report is; how might protective immunity against monkeypox relate to protection against smallpox? Monkeypox serves as an informative surrogate for smallpox in that it is a human pathogen capable of inducing lethal infections in 4–25% of those afflicted (2-6) and smallpox vaccination is cross-protective (3). Applicants examined the immune responses and clinical outcome of subjects infected with a West African strain of monkeypox, which may or may not exhibit the same mortality rates observed in previous monkeypox outbreaks. There are many factors that play a role in monkeypox-induced mortality including the strain of virus involved, the route of infection, the age, nutritional status, immune status and vaccination status of the host, and access to sophisticated medicinal care (13). In our study, we identified 5 vaccinated subjects who contracted monkeypox and 3 vaccinated subjects who demonstrated full protection against the onset of monkeypox-induced disease. The results broadly indicate that almost half of vaccinated individuals (3/8) maintain long-term protective immunity against monkeypox. Interestingly, previous analysis of vaccinia-specific antibody levels in >300 vaccinees showed that ~50% have neutralizing antibody titers of $\geq$1:32 (14) which prior reports suggested would provide fully protective immunity against smallpox (25, 26). In an elegant study involving >300 subjects, the overt smallpox attack rate was 68.8% among unvaccinated contacts compared to only 3.2% in vaccinated contacts (27). Remarkably, 55% (78/142) of vaccinated household contacts had clinically inapparent smallpox infections, indicative of pre-

existing, fully protective immunity (27). A main point of this current study is that applicants' findings are consistent with this much larger previous report (27), and demonstrates that some level of protective immunity is likely to exist in contemporary subjects who have received smallpox vaccination in the distant past.

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EXAMPLE VI

(Monkeypox antigens were identified that are diagnostic for both smallpox and monkeypox, and monkeypox antigens were identified that are specific for smallpox)

In this EXAMPLE applicants have used the inventive SABRE platform to identify monkeypox antigens that are diagnostic for both smallpox and monkeypox, and to identify monkeypox antigens that are specific for smallpox.

TABLE 4 shows, according to particular aspects of the present invention, various exemplary monkeypox B21R peptides that are cross-reactive and recognized by smallpox survivors.

TABLE 4. Antibody cross-reactivity among Smallpox survivors

Peptide	Specificity ≥ 90%	Specificity < 90%
*3	X	
*4	X	
*11	X	
*19	X	
*27	X	
*29	X	
*30		X
*32	X	
*35		X
*37	X	
42	X	
*44	X	
*50	X	
*60	X	
*63	X	
*64		X
*65		X
**67		X
*68		X
73	X	
*74	X	
**75		X
*76		X
*78	X	
*79	X	
*80	X	
100	X	
*115	X	
*126		X
*129	X	

Peptide	Specificity ≥ 90%	Specificity < 90%
*131	X	
**132		X
**141		X
*151		X
**152	X	
*155	X	
**159	X	
166	X	
**168	X	
*169	X	
**170		X
172	X	
*174	X	
*177	X	
**178	X	
*180		X
**184		X
*185	X	
*186	X	

Peptides were designed based on Monkeypox B21R protein sequence.
Specificity is based on 20 naive or vaccinia immune subjects.
*Smallpox international serum standard (pool of 63 subjects, approximately 1 month post-smallpox infection).
**Smallpox international serum standard AND smallpox survivors.

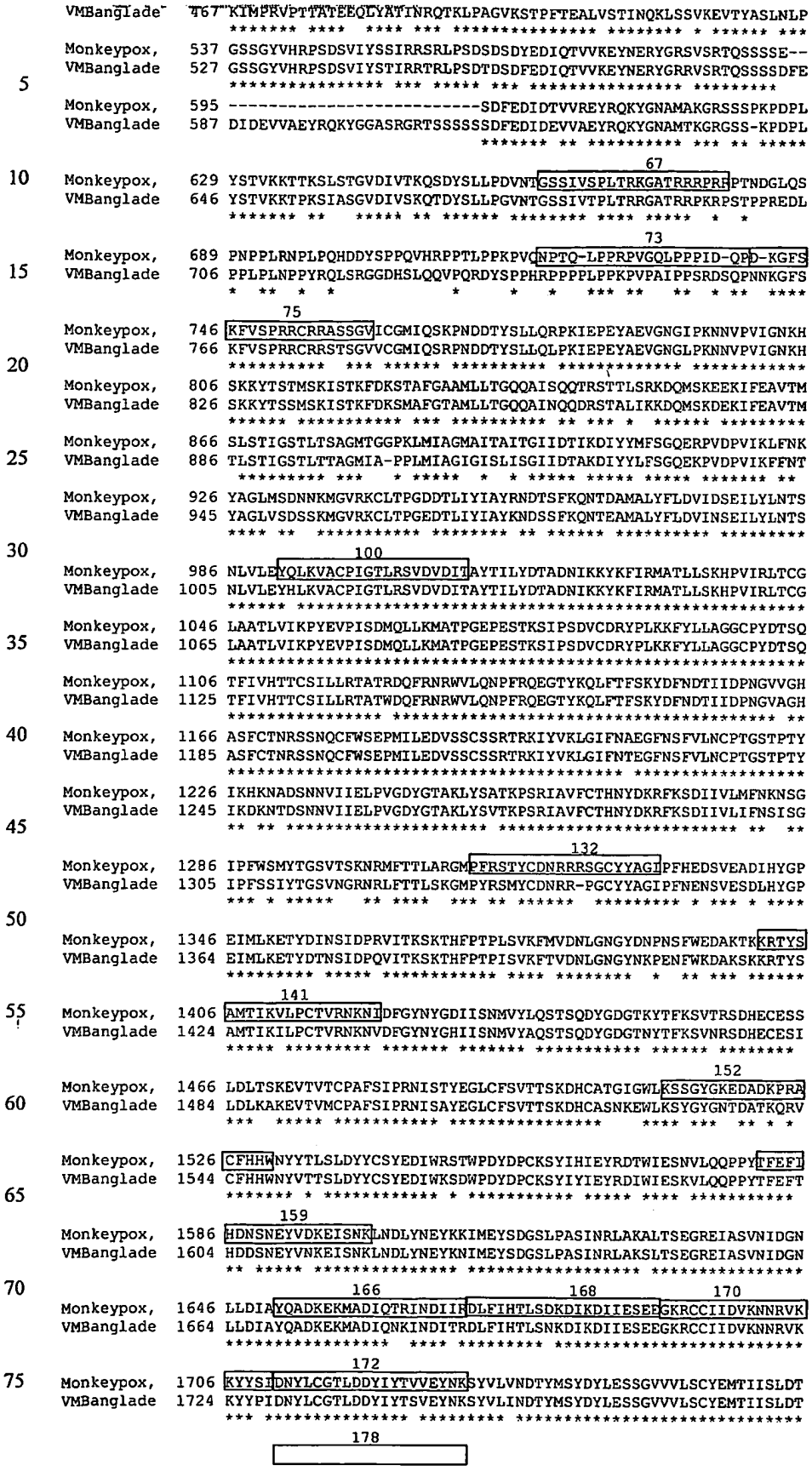
TABLE 5 shows, according to particular aspects of the present invention, various exemplary peptides that are diagnostic for both smallpox and monkeypox.

TABLE 5. Monkeypox B21R/ Variola Major (Bangladesh) B22R alignment; 84.3% identity in 1914 residues overlap. Numbered boxes represent peptides (designed using Monkeypox B21R sequence) for which both Monkeypox and Smallpox survivors have antibody specificity.

```
Monkeypox       1 MNLQKLSLAIYLTVTCSWCYETCMRKTALYHDIQLEHVEDNKDSVASLPYKYLQVVKQRE
VMBanglade      1 MNLQRLSLAIYLTVTCSWCYETCMRKTALFHDNQLGHAEDNQDSVASLPYKYLQVVNKRE
                  **** ************************ ** ** * *** ************** **
Monkeypox,     61 RSRLLATFNWTDIAEGVRNEFIKICDINGTYLYNYTIDVSIIIDSTEELPTVTPITTYEP
VMBanglade     61 RSRLLATFNWTSIAEGVKNDFIRICDINGTYLYNYTIAVSMIIDSMEELPT---ITTYEP
                  *********** ***** * ** *************** ** **** *****   ******
Monkeypox,    121 SIYNYTIDYSTVITTEELQVTP---TYAPVTTPLPTSAVPYDQRSNNNVSTISIQILSKI
VMBanglade    118 STYNYTFDNSTVSTTEELKVTPSPTTYATVTTPLPTSSVPYDQRSNNNVSTISIQILSKI
                  * **** * *** ***** ***   *** ******** **********************

Monkeypox,    178 LGVNETELTNYLIMHKNDTVDNNTMVDDETSDNNTLHGNIGFLEINNCYNVSVSDASFRI
VMBanglade    178 LGVNETELTNYLITHKNATVDNNT----------LYGNIGFLEINNCYNISVSNASFRI
                  ************* *** ******          * ************* *** *****
Monkeypox,    238 TLVNDTSEEILLMLTGTSSSDTFISSTNITECLKTLINNVS-INDVLITQNMNVTSNCDK
VMBanglade    227 TLVNNTSEEIVIMLTGTSSSDTFISSTNITECLKTLINNTSNISDVSITQNMNVTSNCDK
                  **** *****  ***************************** * * ** *************
Monkeypox,    297 CSMNLMASVIPAVNEFNNTLMKIGVKDDENNTVYNYYICKLTTNSTCDELINLDEVINNI
VMBanglade    287 CSMNLMTSVIPAVKEFNNTLKKIGVKDDKNNTVYNYYNCKLTTNSTCDELINLDEVINNI
                  ****** ****** ****** ******* ******** **********************

Monkeypox,    357 TLTNIIRNSVSTTNSRKRRDLNGEFEFSTSKELDCLYESYGVNDDISHCFASPRRRRSDD
VMBanglade    347 TLTNIISSSVSTTNSRKRRDLNGEFEFSTSEELDCLYKSYGVSDDVSHCFSSPRRRRSDD
                  ******  ********************** ****** **** ** **** *********
                          42
Monkeypox,    417 KKEYMDMKLFDHAKKDLGIDSVIPRGTTHFQVGASGASGGVVGDSFPFQNVKSRASLLAE
VMBanglade    407 KQEYTEMKLLDHAKKDLRIDSVIPRGTTHFQVGASGASGGVVGDSSPFQNVKSRASLLAE
                  * **  *** ******* ***************************** **************
Monkeypox,    477 KIMPRVPITATEADLYATVNRQPKLPAGVKSTPFTEALASTINQKLSNVREVTYASSNLP
```


VMBanglade 767 KIMPRVPTTATEEQLYATINRQTKLPAGVKSTPFTEALVSTINQKLSSVKEVTYASLNLP
Monkeypox, 537 GSSGYVHRPSDSVIYSSIRRSRLPSDSDSDYEDIQTVVKEYNERYGRSVSRTQSSSSE--
VMBanglade 527 GSSGYVHRPSDSVIYSTIRRTRLPSDTDSDFEDIQTVVKEYNERYGRRVSRTQSSSSDFE
Monkeypox, 595 ----------SDFEDIDTVVREYRQKYGNAMAKGRSSSPKPDPL
VMBanglade 587 DIDEVVAEYRQKYGGASRGRTSSSSSSDFEDIDEVVAEYRQKYGNAMTKGRGSS-KPDPL
67
Monkeypox, 629 YSTVKKTTKSLSTGVDIVTKQSDYSLLPDVNTGSSIVSPLTRKGATRRRPRHPTNDGLQS
VMBanglade 646 YSTVKKTPKSIASGVDIVSKQTDYSLLPGVNTGSSIVTPLTRRGATRRPKRPSTPPREDL
73
Monkeypox, 689 PNPPLRNPLPQHDDYSPPQVHRPPTLPPKPVQNPTQ-LPPRPVGQLPPPID-QPD-KGFS
VMBanglade 706 PPLPLNPPYRQLSRGGDHSLQQVPQRDYSPPHRPPPPLPPKPVPAIPPSRDSQPNNKGFS
75
Monkeypox, 746 KFVSPRRCRRASSGVICGMIQSKPNDDTYSLLQRPKIEPEYAEVGNGIPKNNVPVIGNKH
VMBanglade 766 KFVSPRRCRRSTSGVVCGMIQSRPNDDTYSLLQLPKIEPEYAEVGNGLPKNNVPVIGNKH
Monkeypox, 806 SKKYTSTMSKISTKFDKSTAFGAAMLLTGQQAISQQTRSTTLSRKDQMSKEEKIFEAVTM
VMBanglade 826 SKKYTSSMSKISTKFDKSMAFGTAMLLTGQQAINQQDRSTALIKKDQMSKDEKIFEAVTM
Monkeypox, 866 SLSTIGSTLTSAGMTGGPKLMIAGMAITAITGIIDTIKDIYYMFSGQERPVDPVIKLFNK
VMBanglade 886 TLSTIGSTLTTAGMIA-PPLMIAGIGISLISGIIDTAKDIYYLFSGQEKPVDPVIKFFNT
Monkeypox, 926 YAGLMSDNNKMGVRKCLTPGDDTLIYIAYRNDTSFKQNTDAMALYFLDVIDSEILYLNTS
VMBanglade 945 YAGLVSDSSKMGVRKCLTPGEDTLIYIAYKNDSSFKQNTEAMALYFLDVINSEILYLNTS
100
Monkeypox, 986 NLVLEYQLKVACPIGTLRSVDVDITAYTILYDTADNIKKYKFIRMATLLSKHPVIRLTCG
VMBanglade 1005 NLVLEYHLKVACPIGTLRSVDVDITAYTILYDTADNIKKYKFIRMATLLSKHPVIRLTCG
Monkeypox, 1046 LAATLVIKPYEVPISDMQLLKMATPGEPESTKSIPSDVCDRYPLKKFYLLAGGCPYDTSQ
VMBanglade 1065 LAATLVIKPYEVPISDMQLLKMATPGEPESTKSIPSDVCDRYPLKKFYLLAGGCPYDTSQ
Monkeypox, 1106 TFIVHTTCSILLRTATRDQFRNRWVLQNPFRQEGTYKQLFTFSKYDFNDTIIDPNGVVGH
VMBanglade 1125 TFIVHTTCSILLRTATWDQFRNRWVLQNPFRQEGTYKQLFTFSKYDFNDTIIDPNGVAGH
Monkeypox, 1166 ASFCTNRSSNQCFWSEPMILEDVSSCSSRTRKIYVKLGIFNAEGFNSFVLNCPTGSTPTY
VMBanglade 1185 ASFCTNRSSNQCFWSEPMILEDVSSCSSRTRKIYVKLGIFNTEGFNSFVLNCPTGSTPTY
Monkeypox, 1226 IKHKNADSNNVIIELPVGDYGTAKLYSATKPSRIAVFCTHNYDKRFKSDIIVLMFNKNSG
VMBanglade 1245 IKDKNTDSNNVIIELPVGDYGTAKLYSVTKPSRIAVFCTHNYDKRFKSDIIVLIFNSISG
132
Monkeypox, 1286 IPFWSMYTGSVTSKNRMFTTLARGMPFRSTYCDNRRRSGCYYAGIPFHEDSVEADIHYGP
VMBanglade 1305 IPFSSIYTGSVNGRNRLFTTLSKGMPYRSMYCDNRR-PGCYYAGIPFNENSVESDLHYGP
Monkeypox, 1346 EIMLKETYDINSIDPRVITKSKTHFPTPLSVKFMVDNLGNGYDNPNSFWEDAKTKKRTYS
VMBanglade 1364 EIMLKETYDTNSIDPQVITKSKTHFPTPISVKFTVDNLGNGYNKPENFWKDAKSKKRTYS
141
Monkeypox, 1406 AMTIKVLPCTVRNKNIDFGYNYGDIISNMVYLQSTSQDYGDGTKYTFKSVTRSDHECESS
VMBanglade 1424 AMTIKILPCTVRNKNVDFGYNYGHIISNMVYAQSTSQDYGDGTNYTFKSVNRSDHECESI
152
Monkeypox, 1466 LDLTSKEVTVTCPAFSIPRNISTYEGLCFSVTTSKDHCATGIGWLKSSGYGKEDADKPRA
VMBanglade 1484 LDLKAKEVTVMCPAFSIPRNISAYEGLCFSVTTSKDHCASNKEWLKSYGYGNTDATKQRV
Monkeypox, 1526 CFHHWNYYTLSLDYYCSYEDIWRSTWPDYDPCKSYIHIEYRDTWIESNVLQQPPYTFEFT
VMBanglade 1544 CFHHWNYVTTSLDYYCSYEDIWKSDWPDYDPCKSYIYIEYRDIWIESKVLQQPPYTFEFT
159
Monkeypox, 1586 HDNSNEYVDKEISNKLNDLYNEYKKIMEYSDGSLPASINRLAKALTSEGREIASVNIDGN
VMBanglade 1604 HDDSNEYVNKEISNKLNDLYNEYKNIMEYSDGSLPASINRLAKSLTSEGREIASVNIDGN
166 168 170
Monkeypox, 1646 LLDIAYQADKEKMADIQTRINDIIRDLFIHTLSDKDIKDIIESEEGKRCCIIDVKNNRVK
VMBanglade 1664 LLDIAYQADKEKMADIQNKINDITRDLFIHTLSNKDIKDIIESEEGKRCCIIDVKNNRVK
172
Monkeypox, 1706 KYYSIDNYLCGTLDDYIYTVVEYNKSYVLVNDTYMSYDYLESSGVVVLSCYEMTIISLDT
VMBanglade 1724 KYYPIDNYLCGTLDDYIYTSVEYNKSYVLINDTYMSYDYLESSGVVVLSCYEMTIISLDT
178

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Monkeypox,  1766 KDAKDAIEDVIVASAVAEALNDMFKEFDKNVSAIIIKEEDNYLNSSPDIYHIIYIIGGTI
VMBanglade  1784 KDAKDAIEDEIVASAVAEALNDMFKEFDKNVSVIIIKEEDNYLNSSPNIYHIIYIIGGTI
                 ********* ********************** ************** ************

                                  184
Monkeypox,  1826 LLLLVIILILAIYIARNKYRTRKYEIMKYDNMSIKSDHHDSLETVSMEIIDNRY
VMBanglade  1844 LILLVIILILVIYIACNKYRTRKYKIMKDDTMSIKSEHHNSLETVSMEIMDNRY
                 * ******** **** ******** *** * ***** ** ********* ****
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TABLE 6 lists multiple (187 different B21R peptides) exemplary monkeypox B21R peptides that are, according to particular aspects of the present invention, diagnostic for both smallpox and monkeypox. The stars indicate how "good" the peptides work by indicating specificity. 90% specificity means, for example, that 18/20 negative controls do not respond to the peptide, and 100% specificity means, for example, that none of the negative controls responded to the specific peptide.

TABLE 6. Exemplary peptides of monkeypox B21R protein

	Peptides	SEQ ID NO.		Hydro	MolWt
1	H- MNLQKLSLAIYLTVTCSWCY	45	-OH	0.70*	2,350.87
2	H- YLTVTCSWCYETCMRKTALY	46	-OH	0.57	2,435.91
3	H- ETCMRKTALYHDIQLEHVED	47	-OH	0.17	2,431.75
4	H- HDIQLEHVEDNKDSVASLPY	48	-OH	0.12	2,309.49
5	H- NKDSVASLPYKYLQVVKQRE	49	-OH	0.08	2,365.73
6	H- KYLQVVKQRERSRLLATFNW	50	-OH	0.21	2,536.00
7	H- RSRLLATFNWTDIAEGVRNE	51	-OH	0.16	2,348.62
8	H- TDIAEGVRNEFIKICDINGT	52	-OH	0.22	2,208.49
9	H- FIKICDINGTYLYNYTIDVS	53	-OH	0.51	2,355.71
10	H- YLYNYTIDVSIIIDSTEELP	54	-OH	0.49	2,361.64
11	H- IIIDSTEELPTVTPITTYEP	55	-OH	0.46	2,232.53
12	H- TVTPITTYEPSIYNYTIDYS	56	-OH	0.43	2,341.57
13	H- SIYNYTIDYSTVITTEELQV	57	-OH	0.40	2,352.59
14	H- TVITTEELQVTPTYAPVTTP	58	-OH	0.42	2,161.45
15	H- TPTYAPVTTPLPTSAVPYDQ	59	-OH	0.40	2,119.37
16	H- LPTSAVPYDQRSNNNVSTIS	60	-OH	0.16	2,163.34
17	H- RSNNNVSTISIQILSKILGV	61	-OH	0.33	2,156.53
18	H- IQILSKILGVNETELTNYLI	62	-OH	0.51	2,274.70
19	H- NETELTNYLIMHKNDTVDNN	63	-OH	0.04	2,378.57
20	H- MHKNDTVDNNTMVDDETSDN	64	-OH	-0.18	2,295.37
21	H- TMVDDETSDNNTLHGNIGFL	65	-OH	0.17	2,193.35
22	H- NTLHGNIGFLEINNCYNVSV	66	-OH	0.39	2,221.49
23	H- EINNCYNVSVSDASFRITLV	67	-OH	0.35	2,244.53
24	H- SDASFRITLVNDTSEEILLM	68	-OH	0.34	2,254.56
25	H- NDTSEEILLMLTGTSSSDTF	69	-OH	0.26	2,161.34
26	H- LTGTSSSDTFISSTNITECL	70	-OH	0.35	2,077.26
27	H- ISSTNITECLKTLINNVSIN	71	-OH	0.37	2,177.52
28	H- KTLINNVSINDVLITQNMNV	72	-OH	0.34	2,243.63
29	H- DVLITQNMNVTSNCDKCSMN	73	-OH	0.23	2,230.56
30	H- TSNCDKCSMNLMASVIPAVN	74	-OH	0.35	2,098.49
31	H- LMASVIPAVNEFNNTLMKIG	75	-OH	0.46	2,162.62
32	H- EFNNTLMKIGVKDDENNTVY	76	-OH	0.07	2,344.60
33	H- VKDDENNTVYNYYICKLTTN	77	-OH	0.13	2,410.66
34	H- NYYICKLTTNSTCDELINLD	78	-OH	0.35	2,336.64
35	H- STCDELINLDEVINNITLTN	79	-OH	0.32	2,234.48
36	H- EVINNITLTNIIRNSVSTTN	80	-OH	0.27	2,216.49
37	H- IIRNSVSTTNSRKRRDLNGE	81	-OH	-0.14	2,316.58
38	H- SRKRRDLNGEFEFSTSKELD	82	-OH	-0.17	2,414.63
39	H- FEFSTSKELDCLYESYGVND	83	-OH	0.23	2,346.53
40	H- CLYESYGVNDDISHCFASPR	84	-OH	0.32	2,276.51
41	H- DISHCFASPRRRRSDDKKEY	85	-OH	-0.16	2,466.74

42	H-	RRRSDDKKEYMDMKLFDHAK	30	-OH	-0.23	2,569.97
43	H-	MDMKLFDHAKKDLGIDSVIP	86	-OH	0.26	2,273.72
44	H-	KDLGIDSVIPRGTTHFQVGA	87	-OH	0.26	2,111.40
45	H-	RGTTHFQVGASGASGGVVGD	88	-OH	0.13	1,859.99
46	H-	SGASGGVVGDSFPFQNVKSR	89	-OH	0.13	1,996.18
47	H-	SFPFQNVKSRASLLAEKIMP	90	-OH	0.32	2,263.71
48	H-	ASLLAEKIMPRVPITATEAD	91	-OH	0.31	2,126.52
49	H-	RVPITATEADLYATVNRQPK	92	-OH	0.14	2,243.56
50	H-	LYATVNRQPKLPAGVKSTPF	93	-OH	0.29	2,187.59
51	H-	LPAGVKSTPFTEALASTINQ	94	-OH	0.32	2,045.34
52	H-	TEALASTINQKLSNVREVTY	95	-OH	0.16	2,237.51
53	H-	KLSNVREVTYASSNLPGSSG	96	-OH	0.10	2,066.27
54	H-	ASSNLPGSSGYVHRPSDSVI	97	-OH	0.20	2,030.20
55	H-	YVHRPSDSVIYSSIRRSRLP	98	-OH	0.20	2,388.73
56	H-	YSSIRRSRLPSDSDSDYEDI	99	-OH	-0.04	2,361.48
57	H-	SDSDSDYEDIQTVVKEYNER	100	-OH	-0.15	2,392.44
58	H-	QTVVKEYNERYGRSVSRTQS	101	-OH	-0.10	2,387.61
59	H-	YGRSVSRTQSSSSESDFEDI	102	-OH	-0.05	2,237.29
60	H-	SSSESDFEDIDTVVREYRQK	103	-OH	-0.10	2,390.52
61	H-	DTVVREYRQKYGNAMAKGRS	104	-OH	-0.12	2,329.64
62	H-	YGNAMAKGRSSSPKPDPLYS	105	-OH	0.06	2,126.39
63	H-	SSPKPDPLYSTVKKTTKSLS	106	-OH	0.08	2,164.50
64	H-	TVKKTTKSLSTGVDIVTKQS	107	-OH	0.07	2,121.47
65	H-	TGVDIVTKQSDYSLLPDVNT	108	-OH	0.24	2,165.40
66	H-	DYSLLPDVNTGSSIVSPLTR	109	-OH	0.33	2,134.39
67	H-	GSSIVSPLTRKGATRRRPRR	31	-OH	-0.09	2,251.64
68	H-	KGATRRRPRRPTNDGLQSPN	110	-OH	-0.24	2,277.55
69	H-	PTNDGLQSPNPPLRNPLPQH	111	-OH	0.18	2,192.43
70	H-	PPLRNPLPQHDDYSPPQVHR	112	-OH	0.17	2,363.64
71	H-	DDYSPPQVHRPPTLPPKPVQ	113	-OH	0.22	2,268.57
72	H-	PPTLPPKPVQNPTQLPPRPV	114	-OH	0.37	2,173.60
73	H-	NPTQLPPRPVGQLPPPIDQP	32	-OH	0.35	2,161.50
74	H-	GQLPPPIDQPDKGFSKFVSP	115	-OH	0.28	2,154.47
75	H-	DKGFSKFVSPRRCRRASSGV	33	-OH	-0.01	2,240.59
76	H-	RRCRRASSGVICGMIQSKPN	116	-OH	0.11	2,219.66
77	H-	ICGMIQSKPNDDTYSLLQRP	117	-OH	0.27	2,279.64
78	H-	DDTYSLLQRPKIEPEYAEVG	118	-OH	0.13	2,323.56
79	H-	KIEPEYAEVGNGIPKNNVPV	119	-OH	0.15	2,167.46
80	H-	NGIPKNNVPVIGNKHSKKYT	120	-OH	0.03	2,208.56
81	H-	IGNKHSKKYTSTMSKISTKF	121	-OH	0.05	2,286.70
82	H-	STMSKISTKFDKSTAFGAAM	122	-OH	0.18	2,109.47
83	H-	DKSTAFGAAMLLTGQQAISQ	123	-OH	0.26	2,038.32
84	H-	LLTGQQAISQQTRSTTLSRK	124	-OH	0.11	2,217.53
85	H-	QTRSTTLSRKDQMSKEEKIF	125	-OH	-0.09	2,413.75
86	H-	DQMSKEEKIFEAVTMSLSTI	126	-OH	0.22	2,287.65
87	H-	EAVTMSLSTIGSTLTSAGMT	127	-OH	0.37	1,958.25
88	H-	GSTLTSAGMTGGPKLMIAGM	128	-OH	0.37	1,881.28
89	H-	GGPKLMIAGMAITAITGIID	129	-OH	0.54	1,943.42
90	H-	AITAITGIIDTIKDIYYMFS	130	-OH	0.58	2,249.67
91	H-	TIKDIYYMFSGQERPVDPVI	131	-OH	0.38	2,371.76
92	H-	GQERPVDPVIKLFNKYAGLM	132	-OH	0.29	2,275.72
93	H-	KLFNKYAGLMSDNNKMGVRK	133	-OH	0.04	2,314.78
94	H-	SDNNKMGVRKCLTPGDDTLI	134	-OH	0.10	2,177.50
95	H-	CLTPGDDTLIYIAYRNDTSF	135	-OH	0.38	2,278.54
96	H-	YIAYRNDTSFKQNTDAMALY	136	-OH	0.19	2,385.66
97	H-	KQNTDAMALYFLDVIDSEIL	137	-OH	0.37	2,299.64
98	H-	FLDVIDSEILYLNTSNLVLE	138	-OH	0.53	2,310.64
99	H-	YLNTSNLVLEYQLKVACPIG	139	-OH	0.50	2,238.65
100	H-	YQLKVACPIGTLRSVDVDIT	34	-OH	0.42	2,191.59
101	H-	TLRSVDVDITAYTILYDTAD	140	-OH	0.32	2,245.48
102	H-	AYTILYDTADNIKKYKFIRM	141	-OH	0.28	2,467.93
103	H-	NIKKYKFIRMATLLSKHPVI	142	-OH	0.37	2,401.02

104	H- ATLLSKHPVIRLTCGLAATL	143	-OH	0.54	2,078.57
105	H- RLTCGLAATLVIKPYEVPIS	144	-OH	0.54	2,144.62
106	H- VIKPYEVPISDMQLLKMATP	145	-OH	0.46	2,273.80
107	H- DMQLLKMATPGEPESTKSIP	146	-OH	0.21	2,173.55
108	H- GEPESTKSIPSDVCDRYPLK	147	-OH	0.09	2,221.49
109	H- SDVCDRYPLKKFYLLAGGCP	148	-OH	0.39	2,245.67
110	H- KFYLLAGGCPYDTSQTFIVH	149	-OH	0.52	2,260.62
111	H- YDTSQTFIVHTTCSILLRTA	150	-OH	0.47	2,270.61
112	H- TTCSILLRTATRDQFRNRWV	151	-OH	0.27	2,437.83
113	H- TRDQFRNRWVLQNPFRQEGT	152	-OH	0.04	2,548.82
114	H- LQNPFRQEGTYKQLFTFSKY	153	-OH	0.24	2,495.84
115	H- YKQLFTFSKYDFNDTIIDPN	154	-OH	0.29	2,469.75
116	H- DFNDTIIDPNGVVGHASFCT	155	-OH	0.34	2,122.31
117	H- GVVGHASFCTNRSSNQCFWS	156	-OH	0.35	2,187.42
118	H- NRSSNQCFWSEPMILEDVSS	157	-OH	0.26	2,329.57
119	H- EPMILEDVSSCSSRTRKIYV	158	-OH	0.25	2,313.70
120	H- CSSRTRKIYVKLGIFNAEGF	159	-OH	0.28	2,289.70
121	H- KLGIFNAEGFNSFVLNCPTG	160	-OH	0.43	2,128.45
122	H- NSFVLNCPTGSTPTYIKHKN	161	-OH	0.26	2,221.54
123	H- STPTYIKHKNADSNNVIIEL	162	-OH	0.18	2,257.54
124	H- ADSNNVIIELPVGDYGTAKL	163	-OH	0.26	2,089.34
125	H- PVGDYGTAKLYSATKPSRIA	164	-OH	0.18	2,095.40
126	H- YSATKPSRIAVFCTHNYDKR	165	-OH	0.14	2,357.69
127	H- VFCTHNYDKRFKSDIIVLMF	166	-OH	0.47	2,476.97
128	H- FKSDIIVLMFNKNSGIPFWS	167	-OH	0.55	2,343.79
129	H- NKNSGIPFWSMYTGSVTSKN	168	-OH	0.22	2,218.49
130	H- MYTGSVTSKNRMFTTLARGM	169	-OH	0.24	2,252.68
131	H- RMFTTLARGMPFRSTYCDNR	170	-OH	0.22	2,423.85
132	H- PFRSTYCDNRRRSGCYYAGI	35	-OH	0.16	2,385.69
133	H- RRSGCYYAGIPFHEDSVEAD	171	-OH	0.14	2,272.46
134	H- PFHEDSVEADIHYGPEIMLK	172	-OH	0.28	2,327.62
135	H- IHYGPEIMLKETYDINSIDP	173	-OH	0.35	2,348.68
136	H- ETYDINSIDPRVITKSKTHF	174	-OH	0.15	2,364.66
137	H- RVITKSKTHFPTPLSVKFMV	175	-OH	0.39	2,316.86
138	H- PTPLSVKFMVDNLGNGYDNP	176	-OH	0.28	2,178.47
139	H- DNLGNGYDNPNSFWEDAKTK	177	-OH	-0.05	2,285.38
140	H- NSFWEDAKTKKRTYSAMTIK	178	-OH	0.03	2,405.78
141	H- KRTYSAMTIKVLPCTVRNKN	36	-OH	0.16	2,323.83
142	H- VLPCTVRNKNIDFGYNYGDI	179	-OH	0.31	2,301.62
143	H- IDFGYNYGDIISNMVYLQST	180	-OH	0.44	2,313.59
144	H- ISNMVYLQSTSQDYGDGTKY	181	-OH	0.18	2,270.47
145	H- SQDYGDGTKYTFKSVTRSDH	182	-OH	-0.07	2,292.42
146	H- TFKSVTRSDHECESSLDLTS	183	-OH	0.09	2,242.42
147	H- ECESSLDLTSKEVTVTCPAF	184	-OH	0.32	2,159.43
148	H- KEVTVTCPAFSIPRNISTYE	185	-OH	0.32	2,255.59
149	H- SIPRNISTYEGLCFSVTTSK	186	-OH	0.33	2,203.52
150	H- GLCFSVTTSKDHCATGIGWL	187	-OH	0.51	2,096.43
151	H- DHCATGIGWLKSSGYGKEDA	188	-OH	0.15	2,095.29
152	H- KSSGYGKEDADKPRACFHHW	37	-OH	0.01	2,319.56
153	H- DKPRACFHHWNYYTLSLDYY	189	-OH	0.38	2,592.90
154	H- NYYTLSLDYYCSYEDIWRST	190	-OH	0.39	2,555.78
155	H- CSYEDIWRSTWPDYDPCKSY	191	-OH	0.32	2,514.75
156	H- WPDYDPCKSYIHIEYRDTWI	192	-OH	0.43	2,600.91
157	H- IHIEYRDTWIESNVLQQPPY	193	-OH	0.38	2,501.80
158	H- ESNVLQQPPYTFEFIHDNSN	194	-OH	0.23	2,379.54
159	H- TFEFIHDNSNEYVDKEISNK	38	-OH	0.03	2,429.60
160	H- EYVDKEISNKLNDLYNEYKK	195	-OH	-0.08	2,505.78
161	H- LNDLYNEYKKIMEYSDGSLP	196	-OH	0.18	2,392.69
162	H- IMEYSDGSLPASINRLAKAL	197	-OH	0.30	2,149.51
163	H- ASINRLAKALTSEGREIASV	198	-OH	0.13	2,086.39
164	H- TSEGREIASVNIDGNLLDIA	199	-OH	0.18	2,087.29
165	H- NIDGNLLDIAYQADKEKMAD	200	-OH	0.06	2,237.49

166	H- YQADKEKMADIQTRINDIIR	39	-OH	0.02	2,421.78
167	H- IQTRINDIIRDLFIHTLSDK	201	-OH	0.31	2,411.80
168	H- DLFIHTLSDKDIKDIIESEE	40	-OH	0.18	2,360.62
169	H- DIKDIIESEEGKRCCIIDVK	202	-OH	0.15	2,306.70
170	H- GKRCCIIDVKNNRVKKYYSI	41	-OH	0.14	2,400.91
171	H- NNRVKKYYSIDNYLCGTLDD	203	-OH	0.10	2,394.66
172	H- DNYLCGTLDDYIYTVVEYNK	42	-OH	0.30	2,401.65
173	H- YIYTVVEYNKSYVLVNDTYM	204	-OH	0.43	2,477.83
174	H- SYVLVNDTYMSYDYLESSGV	205	-OH	0.35	2,305.52
175	H- SYDYLESSGVVVLSCYEMTI	206	-OH	0.49	2,258.57
176	H- VVLSCYEMTIISLDTKDAKD	207	-OH	0.33	2,244.63
177	H- ISLDTKDAKDAIEDVIVASA	208	-OH	0.16	2,074.33
178	H- AIEDVIVASAVAEALNDMFK	43	-OH	0.33	2,106.44
179	H- VAEALNDMFKEFDKNVSAII	209	-OH	0.26	2,254.61
180	H- EFDKNVSAIIIKEEDNYLNS	210	-OH	0.10	2,341.57
181	H- IKEEDNYLNSSPDIYHIIYI	211	-OH	0.34	2,439.72
182	H- SPDIYHIIYIIGGTILLLLV	212	-OH	0.88*	2,226.74
183	H- IGGTILLLLVIILILAIYIA	213	-OH	1.10*	2,108.78
184	H- IILILAIYIARNKYRTRKYE	44	-OH	0.37	2,511.07
185	H- RNKYRTRKYEIMKYDNMSIK	214	-OH	-0.10	2,638.13
186	H- IMKYDNMSIKSDHHDSLETV	215	-OH	0.14	2,363.67
187	H- KSDHHDSLETVSMEIIDNRY	216	-OH	0.05	2,389.60

This Example shows, according to particular aspects, that monkeypox based antigens can be used to provide novel assays that are specific for smallpox. Peptide #67 GSSIVSPLTRKGATRRRPRR (SEQ ID NO:31) was tested on multiple smallpox survivors and found to be a good diagnostic marker that is positive for smallpox but negative for monkeypox. Peptide #67 is recognized by 3/4 smallpox survivors as well as the smallpox international serum standard (pool of 63 blood samples), but is not recognized by monkeypox patients (0/20 samples score positive).

Therefore, according to particular aspects, particular monkeypox antigens can be used to simultaneously detect immunity against smallpox or monkeypox, and can be used to generate antibody reagents for direct detection of both smallpox and monkeyox. According to additional aspects, particular monkeypox antigens can be used to specifically detect smallpox, and can be used to generate antibody reagents for direct and specific detection of smallpox.

CLAIMS

We claim:

1. A high-throughput method for detecting monkeypox virus (MPV) infection, comprising:

-obtaining a test serum sample from a test subject; and

-detecting MPV in the sample using an immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

2. The method of claim 1, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20.

3. The method of claim 2, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

4. The method of claim 2, wherein the MPV protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20.

5. The method of claim 4, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15 and 21-29.

6. The method of claim 5, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), 31, and epitope-bearing fragments of SEQ ID NOS:15 and 27.

7. The method of claim 1, wherein the immunologic assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof.

8. The method of claim 1, wherein the antibody is selected from the group consisting of a single-chain antibody, a monoclonal antibody, a chimeric antibody, a humanized antibody, and a Fab fragment.

9. The method of claim 1, wherein a plurality of antibodies, or eptitope-binding portions thereof, are used, in each case specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

10. A high-throughput method for detecting a monkeypox virus (MPV)-specific immune response, comprising:

-obtaining a test serum sample from a test subject; and

-detecting MPV-specific antibodies in the sample using an immunologic assay, based, at least in part, on use of at least one MPV protein or polypeptide selected from the group consisting of D2L, N2R, N3R, B18R, B21R, epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R, and combinations thereof.

11. The method of claim 10, wherein the monkeypox virus (MPV) protein or polypeptide is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20.

12. The method of claim 11, wherein the MPV polypeptide is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

13. The method of claim 11, wherein the MPV protein or polypeptide is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20.

14. The method of claim 13, wherein the MPV polypeptide is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15 and 21-29.

15. The method of claim 14, wherein the MPV polypeptide is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

16. The method of claim 10, wherein the immunologic assay is selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof.

17. The method of claim 10, wherein a plurality of MPV proteins or polypeptides are used, in each case selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

18. The method of claim 10, wherein detecting monkeypox virus (MPV)-specific antibodies in the sample further comprises determining an amount of MPV-specific antibodies in the sample, and further comprising:

-determining, based at least in part on the amount of MPV-specific antibodies, a corresponding amount of MPV-neutralizing antibodies; thereby providing a determination of a level of *protective immunity* against MPV, based on a historic or contemporaneous correlation between amounts of MPV-neutralizing antibodies and levels of protective immunity against MPV.

19. The method of claim 18, wherein determining the amount of monkeypox virus (MPV)-neutralizing antibodies is by reference to a standard correlation between amounts of MPV-specific antibodies and amounts of MPV-neutralizing antibodies present in serum samples from previously vaccinated or infected individuals.

20. A high-throughput method for parallel detection of both monkeypox virus (MPV) infection and MPV-specific immune response, comprising:

-obtaining a test serum sample from a test subject;

-detecting MPV in the sample using a first immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for an MPV protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R; and

-detecting MPV-specific antibodies in the sample using a second immunologic assay, based, at least in part, on use of at least one of the MPV proteins or polypeptides, thereby providing for detection of both monkeypox virus (MPV) infection and MPV-specific immune response using the same serum sample.

21. The method of claim 18, wherein at least one of the proteins or polypeptides used for detecting MPV-specific antibodies is the cognate antigen of one of the antibody reagents, or epitope binding portions thereof.

22. The method of claim 18, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20.

23. The method of claim 20, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

24. The method of claim 20, wherein the MPV protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20.

25. The method of claim 22, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15 and 21-29.

26. The method of claim 23, wherein the MPV polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

27. The method of claim 18, wherein the first and second immunologic assay is, in each case, selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof.

28. The method of claim 18, wherein the antibody reagent is selected from the group consisting of a single-chain antibody, a monoclonal antibody, a chimeric antibody, a humanized antibody, and a Fab fragment.

29. The method of claim 18, wherein a plurality of antibody reagents, or eptitope-binding portions thereof, are used, and wherein a plurality of MPV protein or polypeptide antigens are used.

30. The method of claim 27, wherein the plurality of antibody reagents, or eptitope-binding portions thereof, and the plurality of MPV protein or polypeptide antigens are cognate pairs.

31. An antibody directed against a monkeypox virus (MPV) protein or polypeptide antigen selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

32. The antibody of claim 31, wherein the antibody is a monoclonal antibody, or antigen-binding portion thereof.

33. The antibody of claim 32, wherein the monoclonal antibody, or antigen-binding portion thereof, is a single-chain antibody, chimeric antibody, humanized antibody or Fab fragment.

34. The antibody of claim 31, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20.

35. The antibody of claim 34, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-44.

36. The antibody of claim 34, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20.

37. The antibody of claim 36, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15 and 21-29.

38. The antibody of claim 37, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

39. A composition, comprising at least one antibody of claim 31.

40. The composition of claim 39, comprising a N3R-specific monoclonal antibody, and a B21R-specific monoclonal antibody.

41. The composition of claim 39, wherein at least one of the antibodies forms specific immunocomplexes with monkeypox whole virions, or proteins or polypeptides associated with monkeypox virions.

42. A pharmaceutical composition, comprising at least one antibody of claim 31, along with a pharmaceutically acceptable diluent, carrier or excipient.

43. The pharmaceutical composition of claim 42, wherein, when administered to a subject, the composition prevents or inhibits monkeypox virus infection.

44. The pharmaceutical composition of claim 42, wherein, when administered to a subject, the composition ameliorates symptoms of monkeypox virus infection.

45. The pharmaceutical composition of claim 42, wherein at least one of the antibodies forms specific immunocomplexes with monkeypox whole virions, or proteins or polypeptides associated with monkeypox virions.

46. A method of treating, or of preventing monkeypox virus infection, comprising administering to a subject in need thereof, a therapeutically effective amount of at least one antibody of claim 1, or of a pharmaceutical composition comprising the antibody.

47. An anti-mokeypox vaccine, comprising at least one monkeypox virus (MPV) protein or polypeptide selected from the group consisting of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

48. The composition of claim 42, wherein the immunoglobulin sequences are, or substantially are, human immunoglobulin sequences.

49. A high-throughput method for parallel detection of both virus infection and immune response against the virus, comprising:

-obtaining a test serum sample from a test subject;

-detecting virus in the sample using a first immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for a viral protein or polypeptide antigen; and

-detecting viral-specific antibodies in the sample using a second immunologic assay, based, at least in part, on use of at least one of the viral proteins or polypeptides, wherein at least

one of the proteins or polypeptides used for detecting virus-specific antibodies is the cognate antigen of one of the antibody reagents, or epitope binding portions thereof.

50. The method of claim 49, wherein the first and second immunologic assay is, in each case, selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof.

51. The method of claim 49, wherein a plurality of antibody reagents, or eptitope-binding portions thereof, are used, and wherein a plurality of viral protein or polypeptide antigens are used.

52. The method of claim 51, wherein the plurality of antibody reagents, or eptitope-binding portions thereof, and the plurality of viral protein or polypeptide antigens are cognate pairs.

53. The method of claim 49, wherein the virus is an orthopoxvirus.

54. The method of claim 53, wherein the orthopoxvirus is selected from the group consisting of smallpox, vaccinia and monkeypox.

55. A high-throughput method for detecting *protective immunity* against smallpox virus, comprising:

-obtaining a test serum sample from a test subject previously vaccinated with a vaccinia-based vaccine;

-detecting an amount of vaccinia virus-specific antibodies in the sample using an immunologic assay; and

-determining, based at least in part on the amount of vaccinia virus-specific antibodies, a corresponding amount of vaccinia virus-neutralizing antibodies; thereby providing a determination of a level of protective immunity against smallpox virus, based on a historic correlation between amounts of vaccinia virus-neutralizing antibodies and protective immunity against small pox virus.

56. The method of claim 55, wherein determining the amount of vaccinia virus-neutralizing antibodies is by reference to a historic or contemporaneous correlation between amounts of vaccinia virus-specific antibodies and amounts of vaccinia virus-neutralizing

antibodies present in serum samples from individuals previously vaccinated with a vaccinia-based vaccine.

57. The method of claim 55, wherein the vaccinia virus-neutralizing antibodies comprise vaccinia intramolecular mature virus (IMV)-neutralizing antibodies.

58. The method of claim 55, wherein the immunologic assay comprises an assay selected from the group consisting of ELISA, immunoprecipitation, immunocytochemistry, Western analysis, antigen capture assays, two-antibody sandwich assays and combinations thereof.

59. The method of claim 55, wherein detecting an amount of vaccinia virus-specific antibodies in the sample using an immunologic assay, comprises forming immunocomplexes between the vaccinia virus-specific antibodies in the sample, and treated vaccinia virus, wherein the vaccinia virus has been treated with a peroxide agent prior to immunocomplex formation.

60. The method of claim 59, wherein the peroxide-treated vaccinia virus is immobilized on a surface prior to immumocomplex formation.

61. The method of claim 59, wherein treating of the vaccinia virus with a peroxide agent comprises treating with hydrogen peroxide.

62. The method of claim 61, wherein, during the treating, the hydrogen peroxide concentration is about 0.5% to about 10%, or about 1.0% to about 5%, or about 2% to about 4%, or about 3% (vol/vol).

63. An array comprising a plurality of different monkeypox virus (MPV) proteins or polypeptides coupled to a solid phase, wherein the MPV proteins or polypeptides are selected from the group consisting of of D2L, N2R, N3R, B18R, B21R and epitope-bearing fragments of D2L, N2R, N3R, B18R and B21R.

64. The array of claim 63, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:1, 6, 10, 16, 20, and epitope-bearing fragments of SEQ ID NOS:1, 6, 10, 16 and 20.

65. The array of claim 64, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29, and epitope bearing fragments of SEQ ID NOS:2-5, 7-9, 11-15, 17-19, 21-29 and 30-34.

66. The array of claim 64, wherein the monkeypox virus (MPV) protein or polypeptide antigen is selected from the group consisting of SEQ ID NOS:10, 20 and epitope-bearing fragments of SEQ ID NOS:10 and 20.

67. The array of claim 66, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:11-15, 21-29 and epitope bearing fragments of SEQ ID NOS:11-15 and 21-29.

68. The array of claim 67, wherein the monkeypox virus (MPV) polypeptide antigen is selected from the group consisting of SEQ ID NOS:15 (MPV $N3R_{157-176}$), 27 (MPV $B21R_{729-748}$), and epitope-bearing fragments of SEQ ID NOS:15 and 27.

69. The array of claim 63, wherein the solid phase comprises a material selected from the group consisting of silicon, cellulose, glass, polystyrene, polyacrylamide, aluminium, steel, iron, copper, nickel, silver, gold and combinations thereof.

70. A high-throughput method for detecting smallpox virus infection, comprising:

-obtaining a test serum sample from a test subject; and

-detecting smallpox in the sample using an immunologic assay based, at least in part, on use of at least one antibody reagent, or epitope-binding portion thereof, specific for an MPV protein or polypeptide antigen selected from the group consisting of B21R and epitope-bearing fragments of B21R.

71. The method of claim 70, wherein the monkeypox virus (MPV) protein or polypeptide antigen comprises SEQ ID NO:31.

72. A high-throughput method for detecting a smallpox virus-specific immune response, comprising:

-obtaining a test serum sample from a test subject; and

-detecting smallpox-specific antibodies in the sample using an immunologic assay, based, at least in part, on use of at least one MPV protein or polypeptide selected from the group consisting of B21R and epitope-bearing fragments of B21R.

73. The method of claim 72, wherein the monkeypox virus (MPV) protein or polypeptide comprises SEQ ID NO:31.

Figure 1a

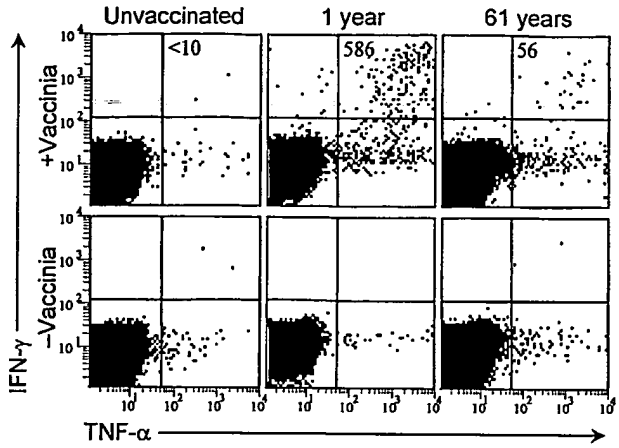
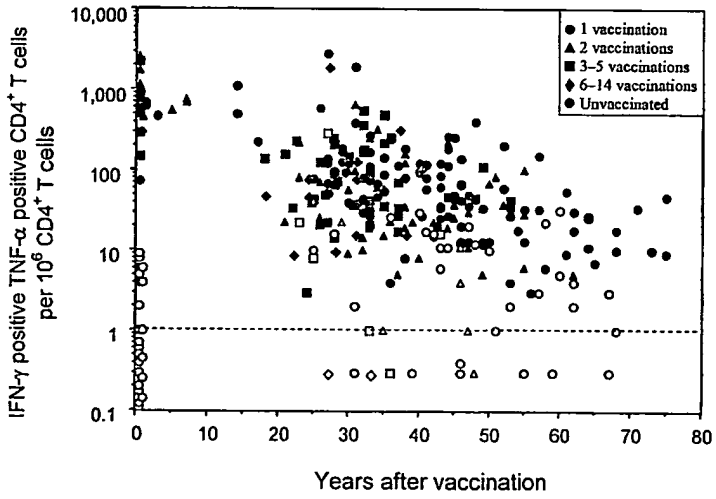


Figure 1b



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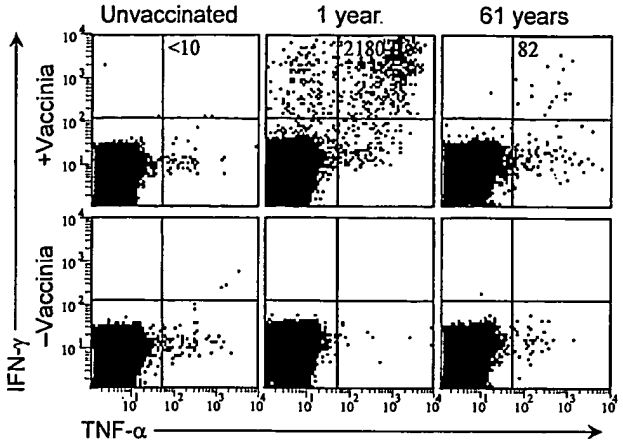
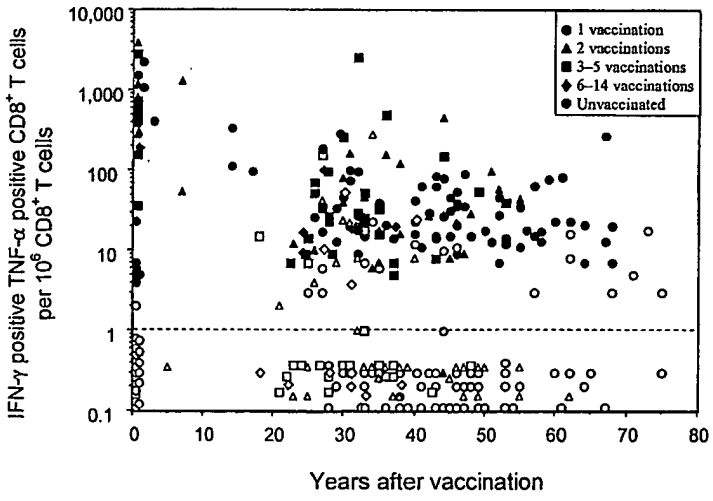


Figure 2b



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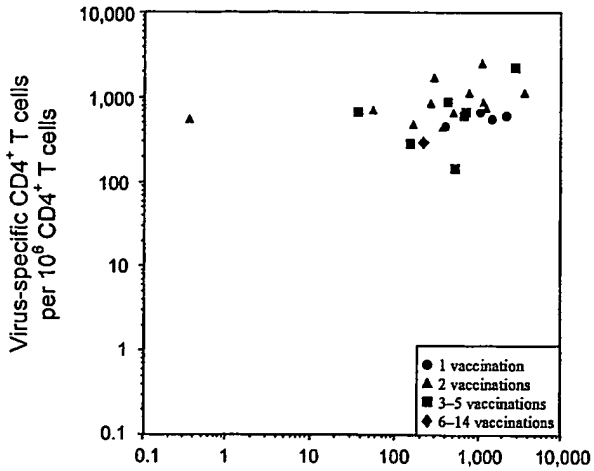


Figure 3b

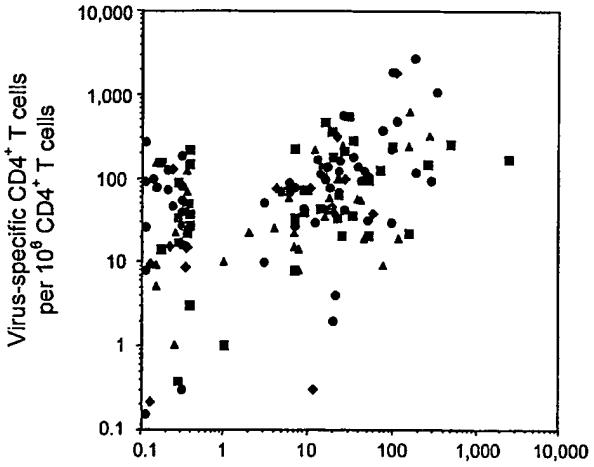


Figure 3c

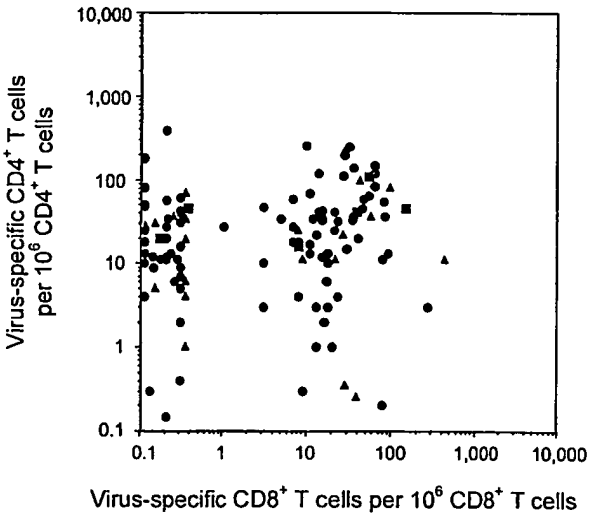
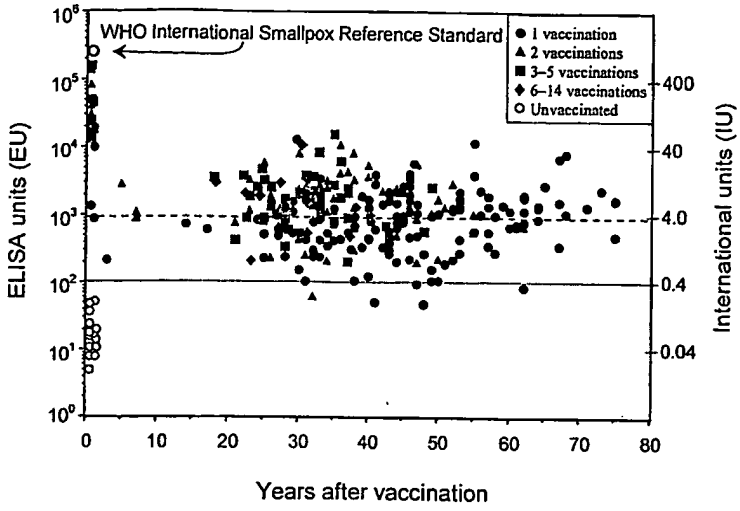


Figure 4a



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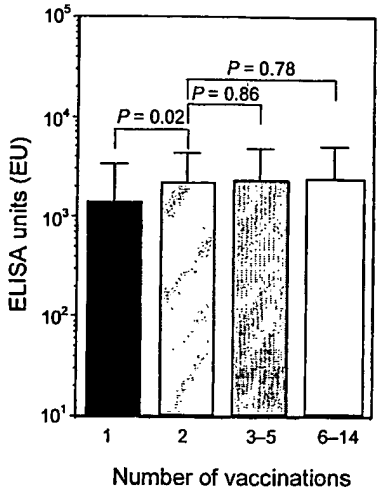

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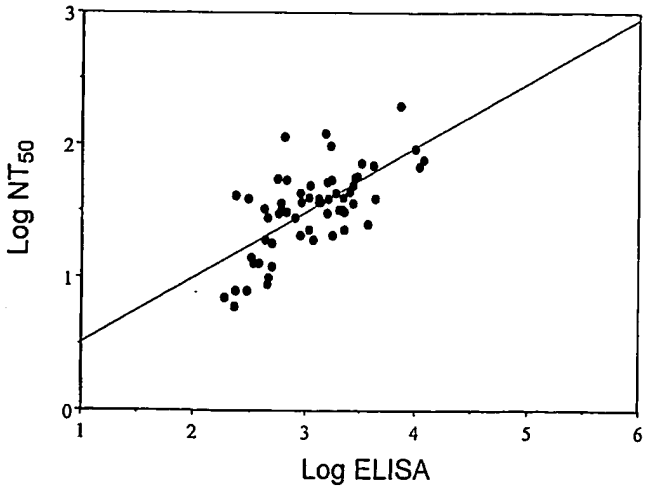

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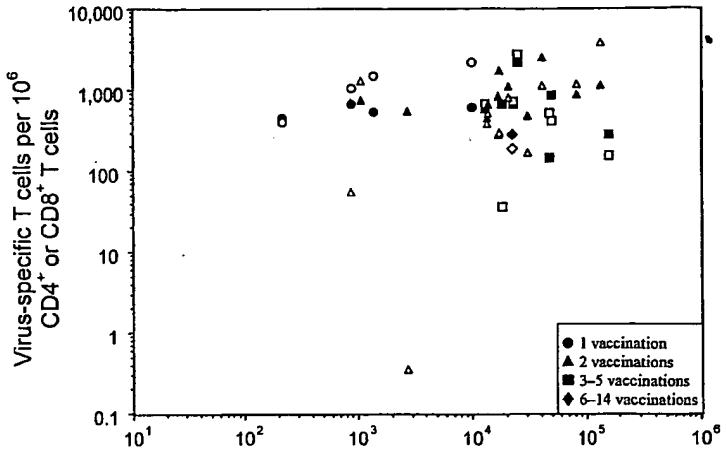

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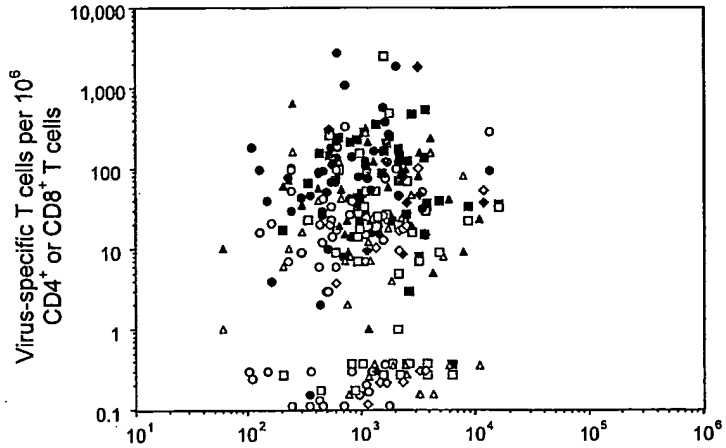
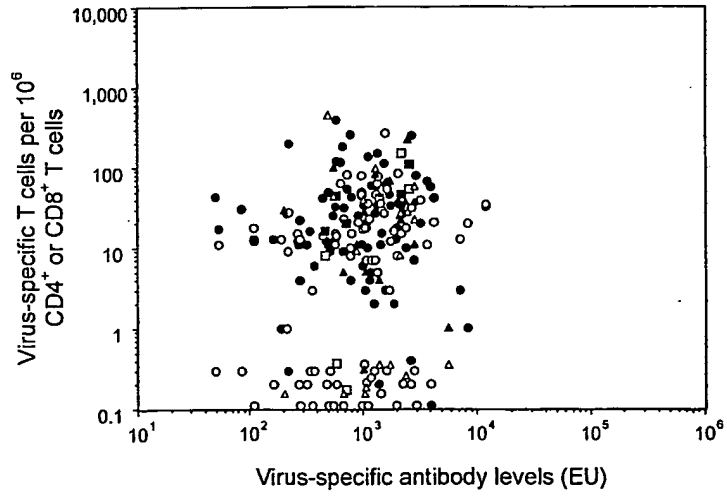


Figure 4f



FIGURES 5A, 5B, 5C, AND 5D

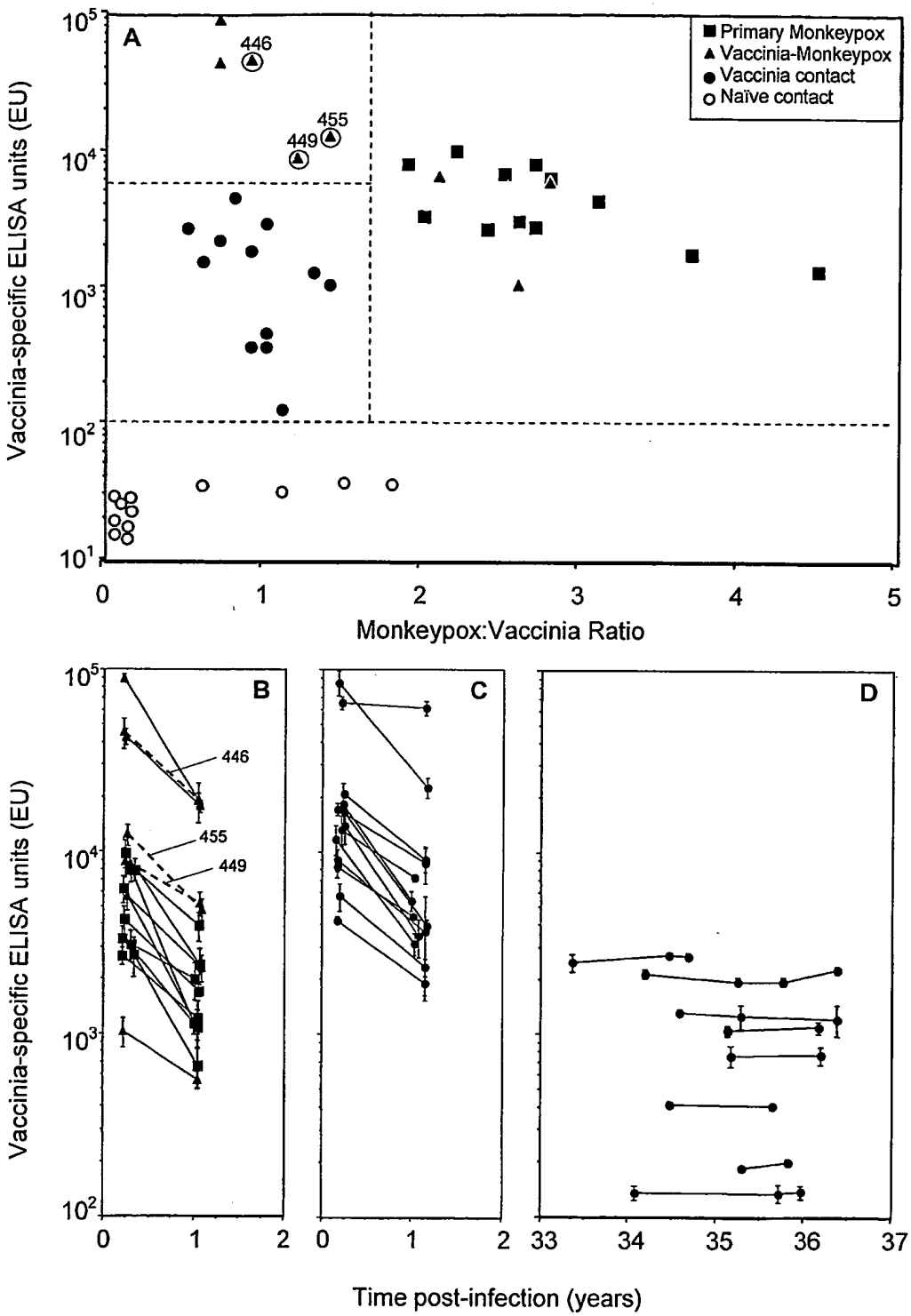
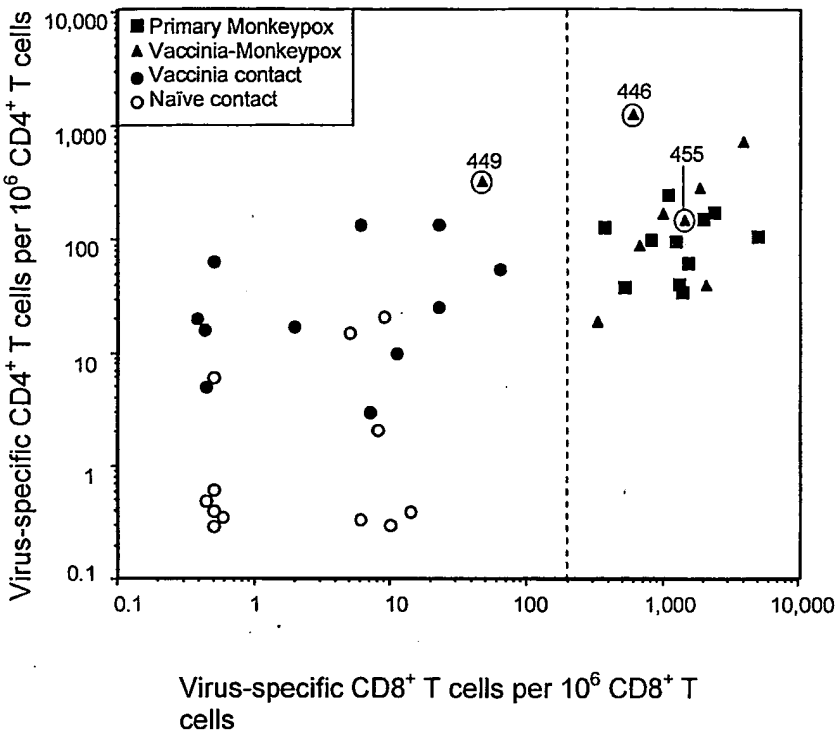


FIGURE 6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FIGURES 7A, 7B, AND 7C

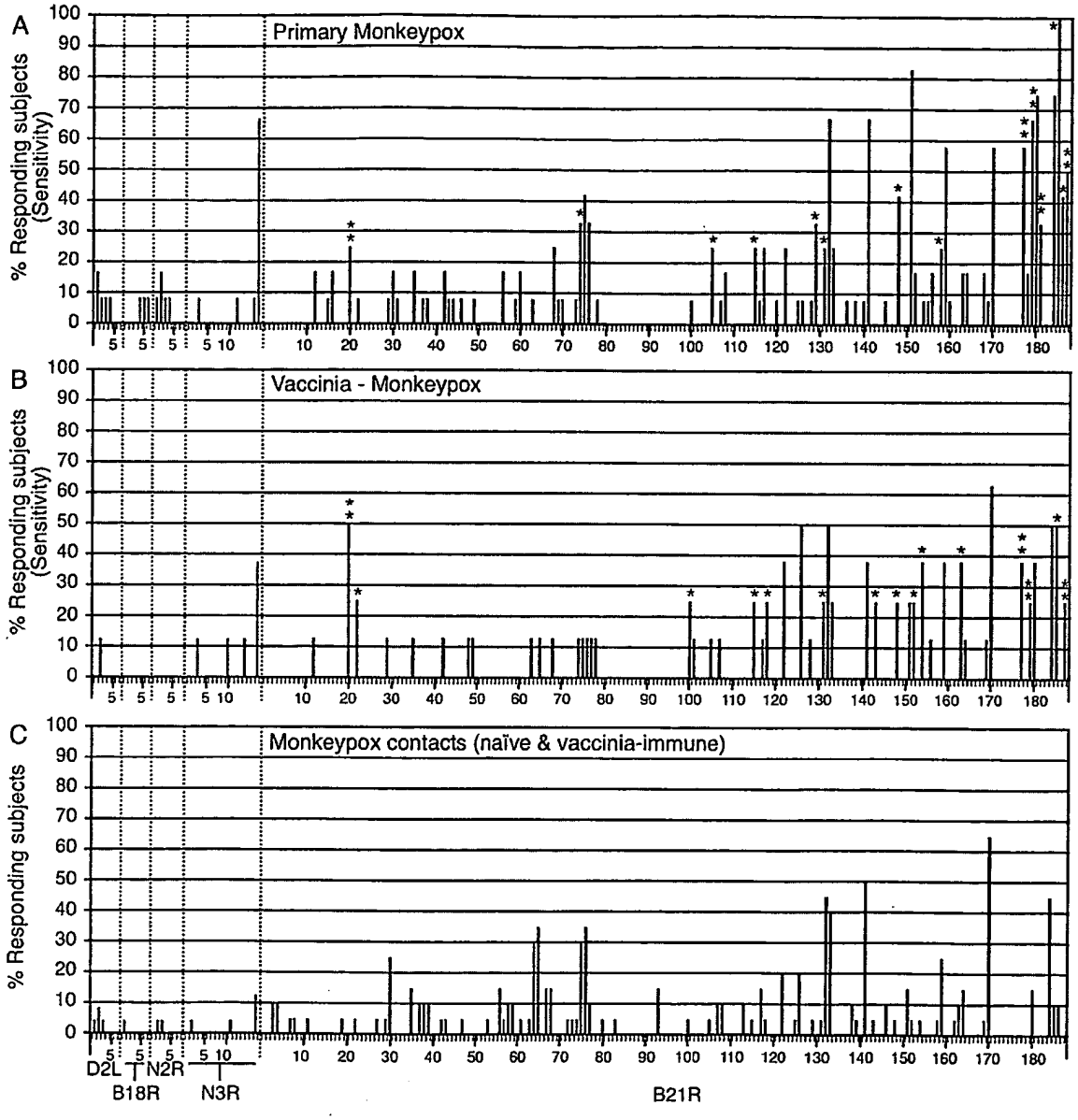
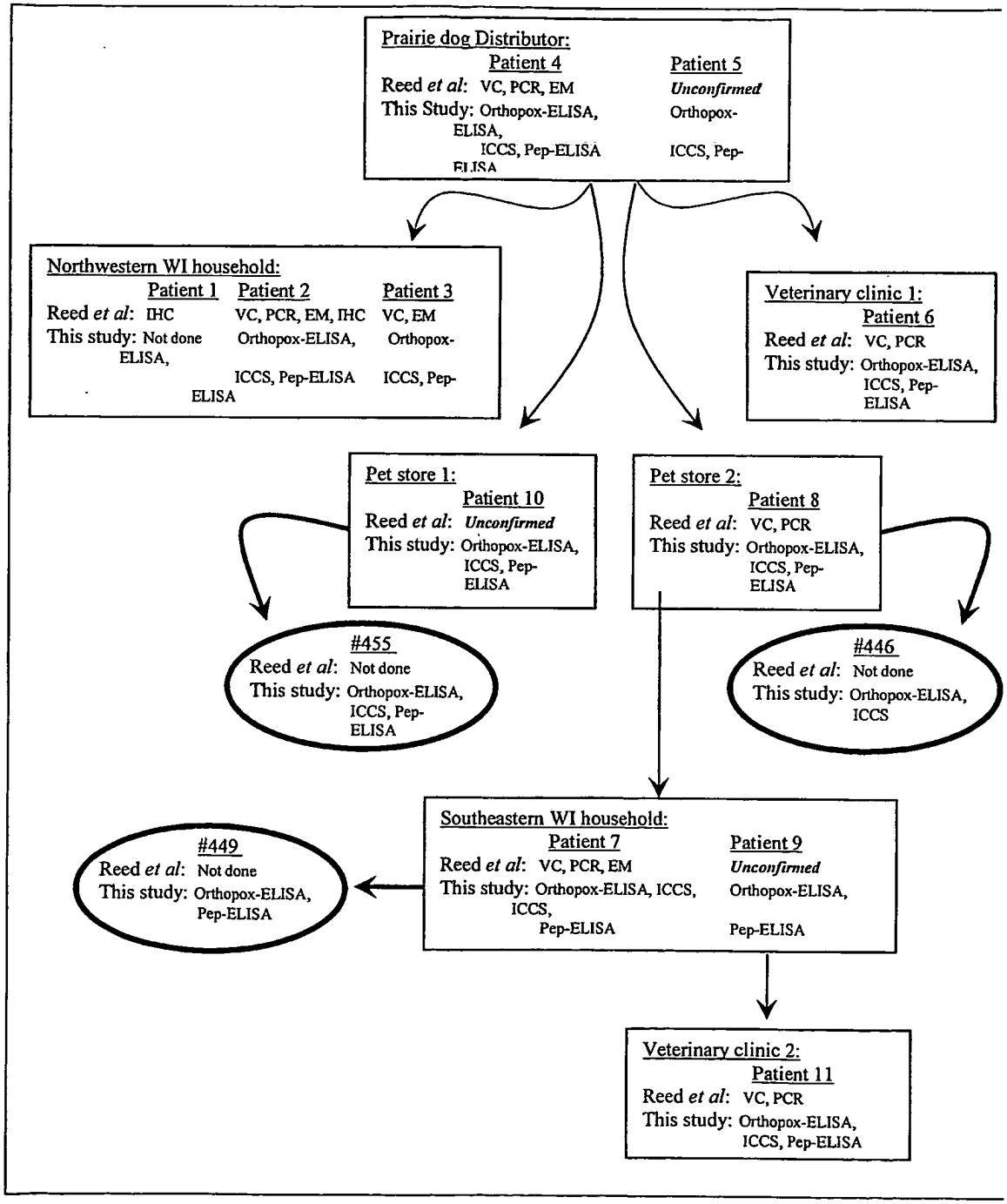


FIGURE 8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Reed et al: VC, PCR, EM
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Reed et al: IHC
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Patient 2
VC, PCR, EM, IHC
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Patient 3
VC, EM
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Veterinary clinic 1:
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Patient 9
Unconfirmed
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Reed et al: VC, PCR
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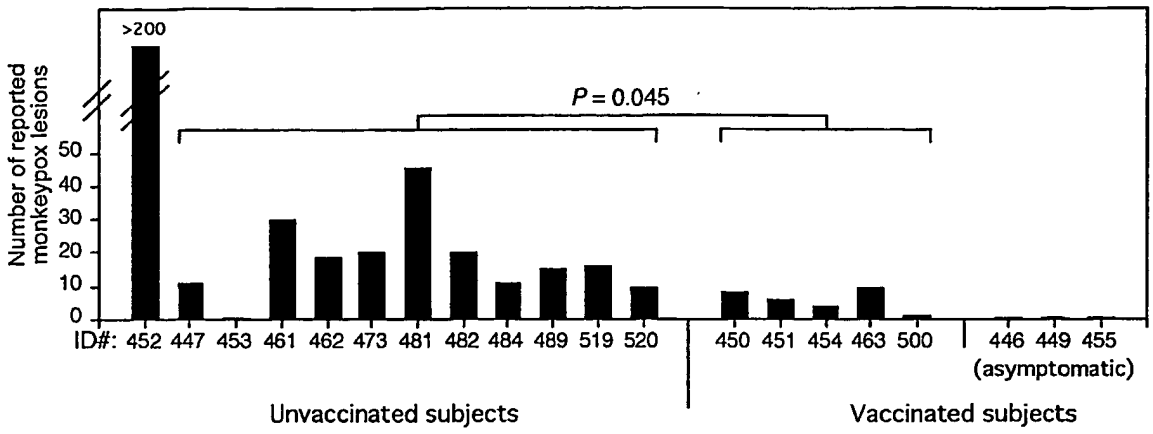
FIGURE 9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Unvaccinated subjects
Vaccinated subjects

SEQUENCE LISTING

<110> Oregon Health & Science University
Slifka, Mark K
Yoshihara, Paul
Hammarlund, Erika

<120> COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF ORTHOPOXVIRUSES

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<150> US 60/579,048
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<213> Monkeypox virus

<400> 9

Arg Tyr Ile Ser His Lys Tyr Pro Leu Cys Asn Ile Ile Asn Gly Tyr
1 5 10 15

Ile Asp Asn Thr
20

<210> 10
<211> 176
<212> PRT
<213> Monkeypox virus

<400> 10

Met Asp Ser Arg Ile Ala Ile Tyr Val Leu Val Ser Ala Ser Leu Leu
1 5 10 15

Tyr Leu Val Asn Cys His Lys Leu Val His Tyr Phe Asn Leu Lys Ile
20 25 30

Asn Gly Ser Asp Ile Thr Asn Thr Ala Asp Ile Leu Leu Asp Asn Tyr
35 40 45

```
Pro Ile Met Thr Phe Asp Gly Lys Asp Ile Tyr Pro Ser Ile Ala Phe
    50                  55                  60

Met Val Gly Asn Lys Leu Phe Leu Asp Leu Tyr Lys Asn Ile Phe Val
65                  70                  75                  80

Glu Phe Phe Arg Leu Phe Arg Val Ser Val Ser Ser Gln Tyr Glu Glu
                85                  90                  95

Leu Glu Tyr Tyr Tyr Ser Cys Asp Tyr Thr Asn Asn Arg Pro Thr Ile
            100                 105                 110

Lys Gln His Tyr Phe Tyr Asn Gly Glu Glu Tyr Thr Glu Ile Asp Arg
        115                 120                 125

Ser Lys Lys Ala Thr Asn Lys Asn Ser Trp Leu Ile Thr Ser Gly Phe
    130                 135                 140

Arg Leu Gln Lys Trp Phe Asp Ser Glu Asp Cys Ile Ile Tyr Leu Arg
145                 150                 155                 160

Ser Leu Val Arg Arg Met Glu Asp Ser Asn Lys Asn Ser Lys Lys Thr
                165                 170                 175
```

<210> 11
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 11

```
Cys His Lys Leu Val His Tyr Phe Asn Leu Lys Ile Asn Gly Ser Asp
1               5                   10                  15

Ile Thr Asn Thr
            20
```

<210> 12
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 12

```
Ser Ser Gln Tyr Glu Glu Leu Glu Tyr Tyr Tyr Ser Cys Asp Tyr Thr
1               5                   10                  15

Asn Asn Arg Pro
            20
```

<210> 13

<211> 20
<212> PRT
<213> Monkeypox virus

<400> 13

Thr Ile Lys Gln His Tyr Phe Tyr Asn Gly Glu Glu Tyr Thr Glu Ile
1 5 10 15

Asp Arg Ser Lys
20

<210> 14
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 14

Asp Ser Glu Asp Cys Ile Ile Tyr Leu Arg Ser Leu Val Arg Arg Met
1 5 10 15

Glu Asp Ser Asn
20

<210> 15
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 15

Ile Tyr Leu Arg Ser Leu Val Arg Arg Met Glu Asp Ser Asn Lys Asn
1 5 10 15

Ser Lys Lys Thr
20

<210> 16
<211> 70
<212> PRT
<213> Monkeypox virus

<400> 16

Met Ile Asp Ile Leu Leu Cys Asp Val Ile Ile Thr Ile Gly Asp Val
1 5 10 15

Glu Ile Lys Ala His Lys Thr Ile Leu Ala Ala Gly Ser Thr Tyr Phe
20 25 30

Lys Thr Met Phe Thr Thr Pro Met Ile Ala Arg Asp Leu Ala Thr Arg
35 40 45

Val Asn Leu Gln Met Phe Asp Lys Met Pro Ser Lys Ile Leu Tyr Ser

50 55 60

Thr Tyr Thr Ile Gly Ile
65 70

<210> 17
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 17

Tyr Phe Lys Thr Met Phe Thr Thr Pro Met Ile Ala Arg Asp Leu Ala
1 5 10 15

Thr Arg Val Asn
20

<210> 18
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 18

Ile Ala Arg Asp Leu Ala Thr Arg Val Asn Leu Gln Met Phe Asp Lys
1 5 10 15

Met Pro Ser Lys
20

<210> 19
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 19

Leu Gln Met Phe Asp Lys Met Pro Ser Lys Ile Leu Tyr Ser Thr Tyr
1 5 10 15

Thr Ile Gly Ile
20

<210> 20
<211> 1879
<212> PRT
<213> Monkeypox virus

<400> 20

Met Asn Leu Gln Lys Leu Ser Leu Ala Ile Tyr Leu Thr Val Thr Cys
1 5 10 15

Ser Trp Cys Tyr Glu Thr Cys Met Arg Lys Thr Ala Leu Tyr His Asp

```
            20                  25                  30

Ile Gln Leu Glu His Val Glu Asp Asn Lys Asp Ser Val Ala Ser Leu
        35                  40                  45

Pro Tyr Lys Tyr Leu Gln Val Val Lys Gln Arg Glu Arg Ser Arg Leu
    50                  55                  60

Leu Ala Thr Phe Asn Trp Thr Asp Ile Ala Glu Gly Val Arg Asn Glu
65                  70                  75                  80

Phe Ile Lys Ile Cys Asp Ile Asn Gly Thr Tyr Leu Tyr Asn Tyr Thr
                85                  90                  95

Ile Asp Val Ser Ile Ile Ile Asp Ser Thr Glu Glu Leu Pro Thr Val
            100                 105                 110

Thr Pro Ile Thr Thr Tyr Glu Pro Ser Ile Tyr Asn Tyr Thr Ile Asp
        115                 120                 125

Tyr Ser Thr Val Ile Thr Thr Glu Glu Leu Gln Val Thr Pro Thr Tyr
    130                 135                 140

Ala Pro Val Thr Thr Pro Leu Pro Thr Ser Ala Val Pro Tyr Asp Gln
145                 150                 155                 160

Arg Ser Asn Asn Asn Val Ser Thr Ile Ser Ile Gln Ile Leu Ser Lys
                165                 170                 175

Ile Leu Gly Val Asn Glu Thr Glu Leu Thr Asn Tyr Leu Ile Met His
            180                 185                 190

Lys Asn Asp Thr Val Asp Asn Asn Thr Met Val Asp Asp Glu Thr Ser
        195                 200                 205

Asp Asn Asn Thr Leu His Gly Asn Ile Gly Phe Leu Glu Ile Asn Asn
    210                 215                 220

Cys Tyr Asn Val Ser Val Ser Asp Ala Ser Phe Arg Ile Thr Leu Val
225                 230                 235                 240

Asn Asp Thr Ser Glu Glu Ile Leu Leu Met Leu Thr Gly Thr Ser Ser
                245                 250                 255

Ser Asp Thr Phe Ile Ser Ser Thr Asn Ile Thr Glu Cys Leu Lys Thr
            260                 265                 270

Leu Ile Asn Asn Val Ser Ile Asn Asp Val Leu Ile Thr Gln Asn Met
```

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        275                 280                 285

Asn Val Thr Ser Asn Cys Asp Lys Cys Ser Met Asn Leu Met Ala Ser
    290                 295                 300

Val Ile Pro Ala Val Asn Glu Phe Asn Asn Thr Leu Met Lys Ile Gly
305                 310                 315                 320

Val Lys Asp Asp Glu Asn Asn Thr Val Tyr Asn Tyr Tyr Ile Cys Lys
                325                 330                 335

Leu Thr Thr Asn Ser Thr Cys Asp Glu Leu Ile Asn Leu Asp Glu Val
            340                 345                 350

Ile Asn Asn Ile Thr Leu Thr Asn Ile Ile Arg Asn Ser Val Ser Thr
        355                 360                 365

Thr Asn Ser Arg Lys Arg Arg Asp Leu Asn Gly Glu Phe Glu Phe Ser
    370                 375                 380

Thr Ser Lys Glu Leu Asp Cys Leu Tyr Glu Ser Tyr Gly Val Asn Asp
385                 390                 395                 400

Asp Ile Ser His Cys Phe Ala Ser Pro Arg Arg Arg Arg Ser Asp Asp
                405                 410                 415

Lys Lys Glu Tyr Met Asp Met Lys Leu Phe Asp His Ala Lys Lys Asp
            420                 425                 430

Leu Gly Ile Asp Ser Val Ile Pro Arg Gly Thr Thr His Phe Gln Val
        435                 440                 445

Gly Ala Ser Gly Ala Ser Gly Gly Val Val Gly Asp Ser Phe Pro Phe
    450                 455                 460

Gln Asn Val Lys Ser Arg Ala Ser Leu Leu Ala Glu Lys Ile Met Pro
465                 470                 475                 480

Arg Val Pro Ile Thr Ala Thr Glu Ala Asp Leu Tyr Ala Thr Val Asn
                485                 490                 495

Arg Gln Pro Lys Leu Pro Ala Gly Val Lys Ser Thr Pro Phe Thr Glu
            500                 505                 510

Ala Leu Ala Ser Thr Ile Asn Gln Lys Leu Ser Asn Val Arg Glu Val
        515                 520                 525

Thr Tyr Ala Ser Ser Asn Leu Pro Gly Ser Ser Gly Tyr Val His Arg
```

```
    530                 535                 540

Pro Ser Asp Ser Val Ile Tyr Ser Ser Ile Arg Arg Ser Arg Leu Pro
545                 550                 555                 560

Ser Asp Ser Asp Ser Asp Tyr Glu Asp Ile Gln Thr Val Val Lys Glu
                565                 570                 575

Tyr Asn Glu Arg Tyr Gly Arg Ser Val Ser Arg Thr Gln Ser Ser Ser
            580                 585                 590

Ser Glu Ser Asp Phe Glu Asp Ile Asp Thr Val Val Arg Glu Tyr Arg
        595                 600                 605

Gln Lys Tyr Gly Asn Ala Met Ala Lys Gly Arg Ser Ser Ser Pro Lys
    610                 615                 620

Pro Asp Pro Leu Tyr Ser Thr Val Lys Lys Thr Thr Lys Ser Leu Ser
625                 630                 635                 640

Thr Gly Val Asp Ile Val Thr Lys Gln Ser Asp Tyr Ser Leu Leu Pro
                645                 650                 655

Asp Val Asn Thr Gly Ser Ser Ile Val Ser Pro Leu Thr Arg Lys Gly
            660                 665                 670

Ala Thr Arg Arg Arg Pro Arg Arg Pro Thr Asn Asp Gly Leu Gln Ser
        675                 680                 685

Pro Asn Pro Pro Leu Arg Asn Pro Leu Pro Gln His Asp Asp Tyr Ser
    690                 695                 700

Pro Pro Gln Val His Arg Pro Pro Thr Leu Pro Pro Lys Pro Val Gln
705                 710                 715                 720

Asn Pro Thr Gln Leu Pro Pro Arg Pro Val Gly Gln Leu Pro Pro Pro
                725                 730                 735

Ile Asp Gln Pro Asp Lys Gly Phe Ser Lys Phe Val Ser Pro Arg Arg
            740                 745                 750

Cys Arg Arg Ala Ser Ser Gly Val Ile Cys Gly Met Ile Gln Ser Lys
        755                 760                 765

Pro Asn Asp Asp Thr Tyr Ser Leu Leu Gln Arg Pro Lys Ile Glu Pro
    770                 775                 780

Glu Tyr Ala Glu Val Gly Asn Gly Ile Pro Lys Asn Asn Val Pro Val
```

785 790 795 800

Ile Gly Asn Lys His Ser Lys Lys Tyr Thr Ser Thr Met Ser Lys Ile
805 810 815

Ser Thr Lys Phe Asp Lys Ser Thr Ala Phe Gly Ala Ala Met Leu Leu
820 825 830

Thr Gly Gln Gln Ala Ile Ser Gln Gln Thr Arg Ser Thr Thr Leu Ser
835 840 845

Arg Lys Asp Gln Met Ser Lys Glu Glu Lys Ile Phe Glu Ala Val Thr
850 855 860

Met Ser Leu Ser Thr Ile Gly Ser Thr Leu Thr Ser Ala Gly Met Thr
865 870 875 880

Gly Gly Pro Lys Leu Met Ile Ala Gly Met Ala Ile Thr Ala Ile Thr
885 890 895

Gly Ile Ile Asp Thr Ile Lys Asp Ile Tyr Tyr Met Phe Ser Gly Gln
900 905 910

Glu Arg Pro Val Asp Pro Val Ile Lys Leu Phe Asn Lys Tyr Ala Gly
915 920 925

Leu Met Ser Asp Asn Asn Lys Met Gly Val Arg Lys Cys Leu Thr Pro
930 935 940

Gly Asp Asp Thr Leu Ile Tyr Ile Ala Tyr Arg Asn Asp Thr Ser Phe
945 950 955 960

Lys Gln Asn Thr Asp Ala Met Ala Leu Tyr Phe Leu Asp Val Ile Asp
965 970 975

Ser Glu Ile Leu Tyr Leu Asn Thr Ser Asn Leu Val Leu Glu Tyr Gln
980 985 990

Leu Lys Val Ala Cys Pro Ile Gly Thr Leu Arg Ser Val Asp Val Asp
995 1000 1005

Ile Thr Ala Tyr Thr Ile Leu Tyr Asp Thr Ala Asp Asn Ile Lys
1010 1015 1020

Lys Tyr Lys Phe Ile Arg Met Ala Thr Leu Leu Ser Lys His Pro
1025 1030 1035

Val Ile Arg Leu Thr Cys Gly Leu Ala Ala Thr Leu Val Ile Lys

1040 1045 1050

Pro Tyr Glu Val Pro Ile Ser Asp Met Gln Leu Leu Lys Met Ala
1055 1060 1065

Thr Pro Gly Glu Pro Glu Ser Thr Lys Ser Ile Pro Ser Asp Val
1070 1075 1080

Cys Asp Arg Tyr Pro Leu Lys Lys Phe Tyr Leu Leu Ala Gly Gly
1085 1090 1095

Cys Pro Tyr Asp Thr Ser Gln Thr Phe Ile Val His Thr Thr Cys
1100 1105 1110

Ser Ile Leu Leu Arg Thr Ala Thr Arg Asp Gln Phe Arg Asn Arg
1115 1120 1125

Trp Val Leu Gln Asn Pro Phe Arg Gln Glu Gly Thr Tyr Lys Gln
1130 1135 1140

Leu Phe Thr Phe Ser Lys Tyr Asp Phe Asn Asp Thr Ile Ile Asp
1145 1150 1155

Pro Asn Gly Val Val Gly His Ala Ser Phe Cys Thr Asn Arg Ser
1160 1165 1170

Ser Asn Gln Cys Phe Trp Ser Glu Pro Met Ile Leu Glu Asp Val
1175 1180 1185

Ser Ser Cys Ser Ser Arg Thr Arg Lys Ile Tyr Val Lys Leu Gly
1190 1195 1200

Ile Phe Asn Ala Glu Gly Phe Asn Ser Phe Val Leu Asn Cys Pro
1205 1210 1215

Thr Gly Ser Thr Pro Thr Tyr Ile Lys His Lys Asn Ala Asp Ser
1220 1225 1230

Asn Asn Val Ile Ile Glu Leu Pro Val Gly Asp Tyr Gly Thr Ala
1235 1240 1245

Lys Leu Tyr Ser Ala Thr Lys Pro Ser Arg Ile Ala Val Phe Cys
1250 1255 1260

Thr His Asn Tyr Asp Lys Arg Phe Lys Ser Asp Ile Ile Val Leu
1265 1270 1275

Met Phe Asn Lys Asn Ser Gly Ile Pro Phe Trp Ser Met Tyr Thr

```
1280                1285                1290

Gly Ser Val Thr Ser Lys Asn Arg Met Phe Thr Thr Leu Ala Arg
1295                1300                1305

Gly Met Pro Phe Arg Ser Thr Tyr Cys Asp Asn Arg Arg Arg Ser
1310                1315                1320

Gly Cys Tyr Tyr Ala Gly Ile Pro Phe His Glu Asp Ser Val Glu
1325                1330                1335

Ala Asp Ile His Tyr Gly Pro Glu Ile Met Leu Lys Glu Thr Tyr
1340                1345                1350

Asp Ile Asn Ser Ile Asp Pro Arg Val Ile Thr Lys Ser Lys Thr
1355                1360                1365

His Phe Pro Thr Pro Leu Ser Val Lys Phe Met Val Asp Asn Leu
1370                1375                1380

Gly Asn Gly Tyr Asp Asn Pro Asn Ser Phe Trp Glu Asp Ala Lys
1385                1390                1395

Thr Lys Lys Arg Thr Tyr Ser Ala Met Thr Ile Lys Val Leu Pro
1400                1405                1410

Cys Thr Val Arg Asn Lys Asn Ile Asp Phe Gly Tyr Asn Tyr Gly
1415                1420                1425

Asp Ile Ile Ser Asn Met Val Tyr Leu Gln Ser Thr Ser Gln Asp
1430                1435                1440

Tyr Gly Asp Gly Thr Lys Tyr Thr Phe Lys Ser Val Thr Arg Ser
1445                1450                1455

Asp His Glu Cys Glu Ser Ser Leu Asp Leu Thr Ser Lys Glu Val
1460                1465                1470

Thr Val Thr Cys Pro Ala Phe Ser Ile Pro Arg Asn Ile Ser Thr
1475                1480                1485

Tyr Glu Gly Leu Cys Phe Ser Val Thr Thr Ser Lys Asp His Cys
1490                1495                1500

Ala Thr Gly Ile Gly Trp Leu Lys Ser Ser Gly Tyr Gly Lys Glu
1505                1510                1515

Asp Ala Asp Lys Pro Arg Ala Cys Phe His His Trp Asn Tyr Tyr
```

1520 1525 1530

Thr Leu Ser Leu Asp Tyr Tyr Cys Ser Tyr Glu Asp Ile Trp Arg
1535 1540 1545

Ser Thr Trp Pro Asp Tyr Asp Pro Cys Lys Ser Tyr Ile His Ile
1550 1555 1560

Glu Tyr Arg Asp Thr Trp Ile Glu Ser Asn Val Leu Gln Gln Pro
1565 1570 1575

Pro Tyr Thr Phe Glu Phe Ile His Asp Asn Ser Asn Glu Tyr Val
1580 1585 1590

Asp Lys Glu Ile Ser Asn Lys Leu Asn Asp Leu Tyr Asn Glu Tyr
1595 1600 1605

Lys Lys Ile Met Glu Tyr Ser Asp Gly Ser Leu Pro Ala Ser Ile
1610 1615 1620

Asn Arg Leu Ala Lys Ala Leu Thr Ser Glu Gly Arg Glu Ile Ala
1625 1630 1635

Ser Val Asn Ile Asp Gly Asn Leu Leu Asp Ile Ala Tyr Gln Ala
1640 1645 1650

Asp Lys Glu Lys Met Ala Asp Ile Gln Thr Arg Ile Asn Asp Ile
1655 1660 1665

Ile Arg Asp Leu Phe Ile His Thr Leu Ser Asp Lys Asp Ile Lys
1670 1675 1680

Asp Ile Ile Glu Ser Glu Glu Gly Lys Arg Cys Cys Ile Ile Asp
1685 1690 1695

Val Lys Asn Asn Arg Val Lys Lys Tyr Tyr Ser Ile Asp Asn Tyr
1700 1705 1710

Leu Cys Gly Thr Leu Asp Asp Tyr Ile Tyr Thr Val Val Glu Tyr
1715 1720 1725

Asn Lys Ser Tyr Val Leu Val Asn Asp Thr Tyr Met Ser Tyr Asp
1730 1735 1740

Tyr Leu Glu Ser Ser Gly Val Val Val Leu Ser Cys Tyr Glu Met
1745 1750 1755

Thr Ile Ile Ser Leu Asp Thr Lys Asp Ala Lys Asp Ala Ile Glu

1760 1765 1770

Asp Val Ile Val Ala Ser Ala Val Ala Glu Ala Leu Asn Asp Met
1775 1780 1785

Phe Lys Glu Phe Asp Lys Asn Val Ser Ala Ile Ile Ile Lys Glu
1790 1795 1800

Glu Asp Asn Tyr Leu Asn Ser Ser Pro Asp Ile Tyr His Ile Ile
1805 1810 1815

Tyr Ile Ile Gly Gly Thr Ile Leu Leu Leu Leu Val Ile Ile Leu
1820 1825 1830

Ile Leu Ala Ile Tyr Ile Ala Arg Asn Lys Tyr Arg Thr Arg Lys
1835 1840 1845

Tyr Glu Ile Met Lys Tyr Asp Asn Met Ser Ile Lys Ser Asp His
1850 1855 1860

His Asp Ser Leu Glu Thr Val Ser Met Glu Ile Ile Asp Asn Arg
1865 1870 1875

Tyr

<210> 21
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 21

Pro Leu Pro Thr Ser Ala Val Pro Tyr Asp Gln Arg Ser Asn Asn Asn
1 5 10 15

Val Ser Thr Ile
20

<210> 22
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 22

Asp Thr Val Asp Asn Asn Thr Met Val Asp Asp Glu Thr Ser Asp Asn
1 5 10 15

Asn Thr Leu His
20

<210> 23
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 23

Ile Arg Asn Ser Val Ser Thr Thr Asn Ser Arg Lys Arg Arg Asp Leu
1 5 10 15

Asn Gly Glu Phe
20

<210> 24
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 24

Thr Arg Lys Gly Ala Thr Arg Arg Arg Pro Arg Arg Pro Thr Asn Asp
1 5 10 15

Gly Leu Gln Ser
20

<210> 25
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 25

Asp Gly Leu Gln Ser Pro Asn Pro Pro Leu Arg Asn Pro Leu Pro Gln
1 5 10 15

His Asp Asp Tyr
20

<210> 26
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 26

Gln His Asp Asp Tyr Ser Pro Pro Gln Val His Arg Pro Pro Thr Leu
1 5 10 15

Pro Pro Lys Pro
20

<210> 27
<211> 20
<212> PRT

<213> Monkeypox virus

<400> 27

Pro Val Gly Gln Leu Pro Pro Pro Ile Asp Gln Pro Asp Lys Gly Phe
1 5 10 15

Ser Lys Phe Val
20

<210> 28
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 28

Lys Asn Asn Val Pro Val Ile Gly Asn Lys His Ser Lys Lys Tyr Thr
1 5 10 15

Ser Thr Met Ser
20

<210> 29
<211> 20
<212> PRT
<213> Monkeypox virus

<400> 29

Thr Arg Ser Thr Thr Leu Ser Arg Lys Asp Gln Met Ser Lys Glu Glu
1 5 10 15

Lys Ile Phe Glu
20

<210> 30
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 42

<400> 30

Arg Arg Arg Ser Asp Asp Lys Lys Glu Tyr Met Asp Met Lys Leu Phe
1 5 10 15

Asp His Ala Lys
20

<210> 31
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 67

<400> 31

Gly Ser Ser Ile Val Ser Pro Leu Thr Arg Lys Gly Ala Thr Arg Arg
1 5 10 15

Arg Pro Arg Arg
20

<210> 32
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 73

<400> 32

Asn Pro Thr Gln Leu Pro Pro Arg Pro Val Gly Gln Leu Pro Pro Pro
1 5 10 15

Ile Asp Gln Pro
20

<210> 33
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 75

<400> 33

Asp Lys Gly Phe Ser Lys Phe Val Ser Pro Arg Arg Cys Arg Arg Ala
1 5 10 15

Ser Ser Gly Val
20

<210> 34
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 100

<400> 34

Tyr Gln Leu Lys Val Ala Cys Pro Ile Gly Thr Leu Arg Ser Val Asp
1 5 10 15

Val Asp Ile Thr
20

<210> 35
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 132

<400> 35

Pro Phe Arg Ser Thr Tyr Cys Asp Asn Arg Arg Arg Ser Gly Cys Tyr
1 5 10 15

Tyr Ala Gly Ile
20

<210> 36
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 141

<400> 36

Lys Arg Thr Tyr Ser Ala Met Thr Ile Lys Val Leu Pro Cys Thr Val
1 5 10 15

Arg Asn Lys Asn
20

<210> 37
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 152

<400> 37

Lys Ser Ser Gly Tyr Gly Lys Glu Asp Ala Asp Lys Pro Arg Ala Cys
1 5 10 15

Phe His His Trp
20

<210> 38
<211> 20
<212> PRT
<213> Artificial sequence

<220>

<223> Monkeypox virus B21R polypeptide 159

<400> 38

Thr Phe Glu Phe Ile His Asp Asn Ser Asn Glu Tyr Val Asp Lys Glu
1 5 10 15

Ile Ser Asn Lys
20

<210> 39
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 166

<400> 39

Tyr Gln Ala Asp Lys Glu Lys Met Ala Asp Ile Gln Thr Arg Ile Asn
1 5 10 15

Asp Ile Ile Arg
20

<210> 40
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 168

<400> 40

Asp Leu Phe Ile His Thr Leu Ser Asp Lys Asp Ile Lys Asp Ile Ile
1 5 10 15

Glu Ser Glu Glu
20

<210> 41
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 170

<400> 41

Gly Lys Arg Cys Cys Ile Ile Asp Val Lys Asn Asn Arg Val Lys Lys
1 5 10 15

Tyr Tyr Ser Ile
20

<210> 42
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 172

<400> 42

Asp Asn Tyr Leu Cys Gly Thr Leu Asp Asp Tyr Ile Tyr Thr Val Val
1 5 10 15

Glu Tyr Asn Lys
20

<210> 43
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 178

<400> 43

Ala Ile Glu Asp Val Ile Val Ala Ser Ala Val Ala Glu Ala Leu Asn
1 5 10 15

Asp Met Phe Lys
20

<210> 44
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Monkeypox virus B21R polypeptide 184

<400> 44

Ile Ile Leu Ile Leu Ala Ile Tyr Ile Ala Arg Asn Lys Tyr Arg Thr
1 5 10 15

Arg Lys Tyr Glu
20